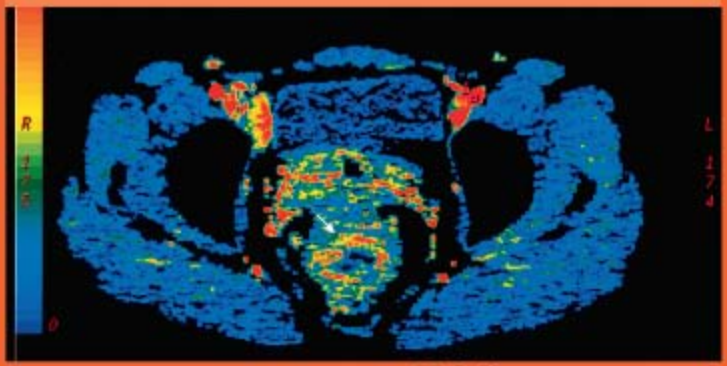
Methods of Cancer Diagnosis, Therapy, and Prognosis

Volume 4
Colorectal Cancer



M.A. Hayat
Editor

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Colorectal Cancer

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Volume 4

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Colorectal Cancer

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New technology, for better or for worse, will be used, as that is our nature.

Lewis Thomas

You have been given the key that opens the gates of heaven; the same key opens the gates of hell.

Writing at the entrance to a Buddhist temple

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Preface

This volume discusses various methodologies for the diagnosis, therapy, and prognosis of colorectal cancer (CRC). Volumes 1, 2, 3, and 4 of this series present similar methods for breast cancer, lung and prostate cancers, and gastrointestinal cancer, respectively. The current volume presents surgical, radiological, and molecular therapies and prognostic biomarkers for CRC. A large number of methods, including immunohistochemistry, *in situ* hybridization, immunoscintigraphy, RT-PCR, free circulating DNA levels in blood and stool, ultrasonography, CT, PET, and MRI are detailed for diagnosing CRC at various stages of development. Imaging technologies for assessing the treatment results are also included as are details related to colonoscopy and sigmoidoscopy. In addition, the role of mircoRNA as a biomarker for this type of cancer is elaborated. Diagnosis of anal squamous cell carcinoma using immunohistochemistry and *in situ* hybridization is also explained. Preoperative staging using endorectal ultrasonography, spectral imaging, and immunohistochemistry is presented, and the usefulness of microarray technology in CRC prognosis is emphasized. The technological advances mentioned above are expected to expedite new discoveries and their translation to clinical practice. Oncology will benefit the most from these various advanced methodologies, as a combination of therapies and personalized medicine will improve early detection of CRC and other cancer types.

The use of anticancer agents, including 5-fluorouracil, fluoropyrimidines, leucovorin, irinotecan, oxaliplatin, raltitrexed, capecitabine, cetuximab, and bevacizumab, is also presented. Preoperative chemoradiotherapy is compared with postoperative chemoradiotherapy. In addition, treatments such as immunotherapy, laparoscopic surgery, radiofrequency ablation, photodynamic therapy, and preoperative chemoradiotherapy, are presented. Preoperative short-course radiation for resectable rectal cancer is also detailed.

This volume included the efforts of 89 contributors representing 19 countries, each a specialist in his or her respective field. Each chapter provides unique, individual, practical knowledge based on the expertise of the authors. I appreciate their dedication and diligent work in sharing their knowledge with the readers. I am also grateful to the authors for their promptness in accepting my suggestions. Strictly uniform style of manuscript writing has been emphasized

throughout. The very high quality of each manuscript made my work as an editor an easy task. Methods presented here also offer a much more detailed, tested information than that available in scientific journals. As with all clinical laboratory testing, results obtained should be interpreted in conjunction with other established and proven laboratory data and clinical findings. The chapters contain the most up-to-date information. Hopefully this volume will be published expeditiously.

I am grateful to Dr. Dawood Farahi and Dr. Kristie Reilly of Kean University for recognizing the importance and necessity of scholarship in an institution of higher education, and for providing the necessary resources to complete this project. I am thankful to Myrna Ortiz, Erin McNally and Betsy Mathew for their help in completing this volume.

M.A. Hayat
September, 2008

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Introduction: Colorectal Cancer

M.A. Hayat

An immense burden is associated with cancer worldwide. This is especially evident among citizens of developing countries. A case in point is that approximately 500,000 women develop cervical cancer each year worldwide, resulting in 250,000 deaths, and 80% of this cancer occurs in developing countries. Many factors, including socio-economic status, cultural perception of malignant conditions, the cost of anticancer chemotherapy, and the paucity of radiation and hospital facilities, (i.e., cancer screening), insufficient transmission of oncology-related information, and, most importantly, inadequate diet, are responsible for the suffering among those in the developing world. Although efforts are being undertaken by private organizations and institutions for reducing the burden of cancer in developing countries, it is evident that governments of developed countries could do much more to address the problem.

Because significant improvements in the prevention and treatment of cardiovascular diseases have occurred, cancer will become the leading cause of death in many parts of the world. In addition, cancer will remain a major health problem, as population aging continues in many countries and elderly persons are more susceptible to this form of malignancy. Approximately, 60% of cancer incidence and 70% of cancer mortality occur among older adults (65 years and older). Unfortunately, it should be noted that cancer care for frail older adults has been mostly unrepresented in clinical trials.

It is estimated that the total number of new colorectal cancer (CRC) cases worldwide and in the United States in 2007 was 1,352,321 and 153,760, respectively. An estimated 52,180 deaths from colon and rectum cancers occurred in 2007 in the United States, accounting for ~ 10% of all cancer deaths. The 1- and 5-year relative survival rate for persons with colorectal cancer is 84% and 64%, respectively. Approximately, 40% of CRCs are diagnosed with localized disease, and stages 0, I, and II show a 5-year survival rate. Prognosis worsens with advancing age. For persons with CRC distant metastasis, the 5-year survival rate is ~ 10%. It should be noted that CRC is the third most common malignancy in both men and women, after lung and breast cancers.

Colorectal cancer (CRC) is the third most common diagnosed cancer globally, and the fourth most frequent cause of

cancer-related deaths, resulting primarily from metastases. Approximately 1 million cases of CRC were diagnosed worldwide in 2006, resulting in > 500,000 deaths. Approximately, 153,700 new cases of this disease (112,340 cases of colon cancer and 41,420 cases of rectal cancer) are diagnosed each year in both sexes in the United States, accompanied by ~ 52,000 deaths, accounting for ~10% of all cancer deaths (Am. CancerSoc., 2007). During the lifetime, a person has 1:8 chance of developing invasive CRC. Although CRC is significantly preventable by detecting and removing premalignant adenomatous polyps, the urgency of early diagnosis is apparent.

Despite advances in surgical techniques, improved chemotherapy and early detection, as stated earlier, CRC is still associated with a relatively poor prognosis. At least 40% of patients who undergo resection of the primary tumor die within 5 years either because of local recurrence or metastatic disease. CRC is, therefore, a major health problem, demanding the elucidication of the mechanism that induces initiation and progression of this neoplasm.

Epidemiological characteristics of colorectal cancer are well recognized. It is known, for example, that environmental factors, specifically dietary and lifestyle factors, play an important role in the etiology of this cancer. Genetic pathways for hereditary and sporadic CRC have been elucidated using advanced technologies in gene analysis. Mutations of high-penetrant genes (e.g., APC) and the family of mismatch repair genes play a major role in the development of hereditary CRC. This gene is associated with familial adenomatous polyposis (Bodmer *et al*., 1987). Mismatch-repair genes are associated with hereditary nonpolyposis colon cancer (Bronner *et al*., 1994). Low penetrant genes contribute to the DNA repair, resulting in a considerable impact on CRC incidence, as they are present in high numbers of the population (de Boer, 2002). Yeh *et al*. (2007) have discussed the role of genes in and risk of CRC.

CRC develops over decades, with progressive accumulation of genetic mutations. Only ~ 15% of CRCs are caused by germline mutations targeting a limited number of genes, including *APC, MLH1, MSH2, MSH3, PMS1, and PMS2* (Booth, 2007). Most CRC cases arise sporadically and involve progression from adenoma to carcinoma. Chromosomal instability and microsatellite instability are genetic abnormalities associated with CRC, which predispose persons to the development of this disease. The former is characterized by mutations in *APC, KRAS, and TP53* along with loss of heterozygosity, while the latter is characterized by mutations in mismatch repair genes including *MLH1 and MSH2*. Hypermethylation of CpG island methylator phenotype has been proposed as a third pathway to CRC (Issa *et al*., 2005).

Histological examination of CRC shows immunoinflammatory cells in the vicinity of these tumors (Simiantonaki *et al*., 2007). This topological arrangement implies a relationship among inflammation, the immune response, and cancer. Tumor microenvironments are thought to underlie this interconnected, dynamic system. Both tumor cells and the peritumoral immunoinflammatory cells produce mediators, such as cytokines, that activate the immune system and control the contact with the endothelium (Coussens and Werb, 2002). However, cytokines play opposing roles in tumorigenesis. They can promote malignant progression or can act as anti-tumor agents (Wilson and Balkwill,

2002). It is known that patients with inflammatory bowel disease are also at high risk for developing colon cancer. Inflammatory bowel diseases are chronic relapsing conditions of unknown etiology; both genetic and environmental factors have been implicated.

SCREENING FOR COLORECTAL CANCER

The most important prognostic factors for CRC are the depth of invasion, lymph node status and distant metastasis. Presently, therapeutic decisions are mainly made on the TNM staging of cancer.

Early detection is necessary to decrease CRC-related mortality, because early-stage disease shows good prognosis and later stages have poor survival rates. A number of methods are available for detecting CRC at an early stage, which provide prognostic and predictive information. Current guidelines recommend fecal-occult blood testing every 1 or 2 years, flexible sigmoidoscopy every 5 years, and colonoscopy every 10 years, usually beginning at age 50 (U.S. Preventive Services Task Force, 2002). In fact, CRC screening is recommended for average risk persons over the age of 50 years. Guaiac-based fecal-occult blood testing (gFOBT) is the only screening method for CRC, which has been demonstrated to reduce mortality. Drawbacks of this method are dietary and drug restrictions and multiple samples are required. The first two drawbacks can be overcome by using immunolo-gical FOBT. However, even this protocol shows poor sensitivity for adenomas and early-stage carcinomas. On the other hand, multi-marker DNA-based stool testing is a promising approach to achieve increased sensitivity (Booth, 2007). The limitation of this method is its high cost. Another recently introduced protocol to increase sensitivity of CRC detection involves the detection of p16 in the serum of patients; this method is termed limiting dilution-methylation-specific polymerase chain reaction (Nakayama *et al*., 2007). Small amounts of tumor DNA can be detected in the serum, and the specificity is confirmed because no abnormal methylation in serum is observed if the corresponding tumor does not exhibit methylation. However, p16 methylation of serum DNA is not specific for CRC.

Colonoscopy is considered to be the gold standard for the detection of colorectal neoplasia, and is the primary screening approach for this cancer. It is a noninvasive, low risk screening method for CRC and the sensitivity of this method has been reported to be 55–92% (Rockey *et al*., 2005). However, a number of studies report that colonoscopy may fail to detect clinically important neoplastic lesions. For example, tandem colonoscopy study has shown a pooled miss rate for polyps of any size as high as 22% (Jeroen *et al*., 2006). According to Imperiali *et al*. (2007), implementing a continuous quality improvement program in routine colonoscopy practice will result in significant improvement in quality performance. The major barrier to colorectal screening is bowel preparation because many patients are often reluctant to undergo laxative preparation. It is estimated that only 40% of the 70 million eligible Americans have had any type of colorectal screening within the recommended screening intervals. Development of a patient-friendly examination will be a major advance.

A number of biomarkers have been identified for the detection and/or prognosis of

CRC in colon tissues, feces, and serum. They provide various levels of accuracy. Molecular-based direct colorectal cancer screening of stool samples has gained considerable attention (Berger *et al*., 2003). The sensitivity of this test (DNA Direct Inc., San Francisco, CA) is fourfold greater than that yielded by FOBT alone (Imperiale *et al*., 2004). Recently, it was reported that plasma levels of different lysophosphatidylcholines (LPCs) forms are significantly reduced in CRC patients compared with unaffected controls, and thus represent useful biomarkers for CRC (Zhao *et al*., 2007). These lipids are important cell signaling molecules, and it has been shown that lysophosphatidic acid is an autocrine growth factor that stimulates proliferation, adhesion, migration, invasion, and tumor metastasis of ovarian cancer cells (Ren *et al*., 2006). However, further validation of the clinical application of LPC levels as a first-line screening method for CRC and the detection of adenomas remains to be universally implemented.

In addition, computed tomography colonoscopy is an option, which is an attractive, noninvasive method. Exfoliated colonocyte analysis can also be used for screening malignant CRC tumors (Loktionov, 2007). Such tumors exfoliate huge amounts of cells that mostly remain within the mucocellular layers overlying colorectal mucosa. Cell containing fragments of the mucocellular layer excreted with stool constitute the main source of exfoliated colonocytes found in human feces. Migration of the cell-containing mucocellular layer toward rectum creates conditions for the accumulation of exfoliated colonocytes on the surface of the rectal mucosa (Loktionov, 2007). However, further studies are required to study colonocyte population dynamics in normal conditions and cancer.

A recent study, although preliminary, indicates that laxative-free colon examination using barium for stool labeling can be performed at computed tomography colonography with or without stool subtraction with accuracy, but further study is warranted to recommend its routine use (Johnson *et al*., 2007).

Recent improvements in chemotherapy have extended survival duration for CRC and other cancer types. For years, advanced CRC therapy was limited to fluorouracil, but success in using individual drugs alone is low. In the 1990s, two additional agents, irinotecan and oxaliplatin, were found to possess activity against advanced CRC therapy. Initial treatment with fluorouracil and irinotecan results in a median survival of ~ 15 months, and second-line therapy with oxaliplatin further improves disease control. It has also been shown that initial treatment with irinotecan and either bolus (North American preference) or infused (European preference) fluorouracil plus leucovorin significantly improved outcomes in CRC patients (Goldberg *et al*., 2006). Irinotecan inhibits topoisomerase 1, impending DNA uncoiling and causing double-strand DNA breaks. The addition of bevacizumab (at 10 mg/kg) to oxaliplatin, fluorouracil, and leucovorin improves survival duration for patients with previously treated metastatic CRC (Giantonio *et al*., 2007). In other words, antiangiogenic therapy with bevacizumab in combination with the above-mentioned drugs, has been demonstrated to prolong the progression-free survival of patients.

As mentioned earlier, application of these drugs in combination shows significant

promise in increasing their effectiveness, but toxicity remains a problem. It has been shown, for example, that combining bevacizumab with oxaliplatin-containing regimen (FOLFOX4) results in a 14% overall increase in grade 3 and 4 toxicity (Giantonio *et al*., 2007). Unfortunately, the side effects include hypertension, bleeding, vomiting, sensory neuropathy, and bowel perforation.

TREATMENT

Surgical resection is the cornerstone of therapy for colon cancer, although this method is still evolving. Indeed, surgery is the mainstay of CRC treatment, with adjuvant chemotherapy and radiotherapy for specific subgroups of patients. The advent of minimally invasive surgical technique, termed laparoscopy, has given surgeons the option for colon resection. Laparoscopy has the advantages of less intraoperative blood loss, decreased postoperative pain, and shorter length of hospitalization. Laparoscopic protocol, however, requires larger operative times than those for the traditional open surgery. Other concerns in the past regarding the former protocol were recurrence of both wound and port site malignancy (Berends *et al*., 1994). Nevertheless, recent studies using refined laparoscopic surgery indicate that this procedure is an oncologically sound option for CRC treatment, and may offer distinct advantages over traditional open surgery (Jackson *et al*., 2007). In spite of the reported advantages and limitations of these two methods, the oncologic outcomes resulting from these two methods must continue to be evaluated.

Because the liver is the most common anatomical site of metastasis by CRC, a brief comment on the prognosis and treatment of such patients is in order. The role of carcinoembryonic antigen (CEA) in the progression of colon cancer cells to the liver is firmly established (Minami *et al*., 2001). CEA, a glycoprotein of ~ 180 kDa, is frequently expressed in colorectal tumors. Although CEA is not an effective worker for screening, it is the most useful indicator for monitoring therapeutic efficacy of surgery in colorectal cancer, as increasing levels of this antigen in the serum after surgery often correlate with either local recurrence or as development of metastasis.

Surgical resection is the standard treatment for patients with resectable colorectal liver metastases. Five year survival rates after resection have been reported to be as high as 58%, especially when hepatic resection is combined with chemotherapy (Pawlik *et al*., 2005). However, only 15–20% of patients with this disease are candidates for surgical resection at the time of diagnosis. The response rate achieved with the combination of 5-FU and leucovorin is only ~ 20%. Combined fluoropyrimidines and irinotecan or oxaliplatin treatment yields response rates of 55% with a median survival of 22 months in patients with stage IV colorectal cancer (Douillard *et al*., 2000). Because chemotherapy-related liver injuries are not uncommon, protecting the liver parenchyma from such treatments remains a serious consideration. Thus, response to treatment should not be the only criterion when selecting chemotherapy for patients with colorectal liver metastases. Zorzi *et al*. (2007) have reviewed liver injury associated with preoperative chemotherapy for colorectal liver metastases.

RECTAL CANCER

Localized adenocarcinoma of the rectum is often curable. Surgery is the major treatment, with a cure rate of ~ 60%. Because of an increased tendency for locoregional failure, pelvic radiation is used routinely to treat patients with stage II or III rectal cancer. Both pre- and postoperative radiotherapy reduces local failure rates and improves survival. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer can be carried out. Combination of preoperative radiotherapy with capecitabine and oxaliplatin for downstaging rectal cancer is discussed in this volume by J. Machiels. Recent evidence indicates that the combination of oxaliplatin, raltitrexed, and fluorouracil/folinic acid with pelvic radiotherapy is recommended for treating locally advanced rectal cancer patients, with acceptable toxicity. Chemotherapy is delivered immediately prior to radiotherapy. The safety of this procedure can be improved by reducing the dose of fluorouracil to 800 mg m^{-2} (Avallone *et al*., 2007).

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A. Diagnosis

2
Poorly Differentiated Colorectal Adenocarcinoma (Methodology)

Seiichi Shinji, Zenya Naito, Toshiyuki Ishiwata, Yoko Matsuda, Tomoko Seya, and Takashi Tajiri

INTRODUCTION

Poorly differentiated adenocarcinoma (PDAC) constitutes ~ 2–25% of all colorectal carcinomas (CRCs) (Riddle *et al*., 2003; Ueno *et al*., 2002). In Japan, the frequency of PDAC among CRCs has been reported to be > 5%, whereas it is between 10–25% in Western countries (Taniyama *et al*., 1991). Clinically, PDAC often penetrates deep through the bowel wall and frequently metastasizes to the lymph nodes or distant organs. PDAC is characterized by local recurrences and/or distant metastases despite curative surgery, and its prognosis is poor compared to well- or moderately-differentiated colorectal adenocarcinoma (Chung *et al*., 1982).

Some PDACs show neuroendocrine cell differentiation in the part of the tumor. Neuroendocrine cell differentiation occurs in about 15% of PDAC patients (Shinji *et al*., 2006). The frequency of liver metastasis at the time of diagnosis is significantly higher in PDAC patients with neuroendocrine cell differentiation than in those without neuroendocrine cell differentiation. The microvessel density and vascular endothelial growth factor-A (VEGF-A) expression level tend to be high in PDAC patients with neuroendocrine cell differentiation, and PDAC with neuroendocrine cell differentiation might induce liver metastasis through microvessel formation in the tumor as induced by VEGF-A. In the near future, the regulation of VEGF-A expression in PDAC patients with or without neuroendocrine cell differentiation might become a new molecular target for the inhibition of liver metastasis and tumor regression.

HISTOPATHOLOGY

PDACs have little or no gland formation; if glands are present, they are small or architecturally complex. Moreover, the invasive part of colorectal carcinoma may occur as single cells or small clusters of cells usually lacking a lumen, the so-called PDAC configuration. Rarely, tumors are dedifferentiated, forming cohesive sheets. In the WHO classification of colorectal carcinoma, it is proposed that the percentage of glandlike structures in the tumor should be used to define the CRC grade. Well-differentiated (grade 1) lesions exhibit glandular structures in > 95% of the tumor; moderately differentiated (grade 2)

adenocarcinoma has 50–95% of glands; PDAC (grade 3) has 5–50% of glands (Hamilton and Aaltonen, 2000). There are various PDACs that show special or specific patterns of differentiation. PDACs often have an appearance reminiscent of poorly differentiated endocrine carcinoma. These PDACs show neuroendocrine cell differentiation in a part of the tumor as determined by immunohistochemical staining for chromogranin A, synaptophysin, neural cell adhesion molecule (NCAM/CD56), and neuron-specific enolase (NSE) (Riddle *et al*., 2003).

In clinical samples, the invasive part of the tumor often shows a low grade of differentiation (PDAC configuration) characterized by the lack of a glandular structure and lumen, although the main tumor is well differentiated. Considering that the invasive front of the tumor is thought to be the most progressive part, the PDAC configuration in a well-differentiated tumor may indicate high invasive and malignant activities. Ueno *et al*. (2002) defined tumor 'budding' (Figure 2.1) as an isolated single cancer cell or a cluster composed of fewer than five cancer cells, and it is associated with prognosis. The histological finding of tumor budding was reported to be one of the important risk factors of an adverse outcome in early invasive CRC and to be related to the risk of nodal involvement (Ueno *et al*., 2004). Gosens *et al*. (2007) reported the relationship between membranous epithelial cell adhesion molecule (Ep-CAM) and budding in CRC. In contrast to the tumor mass of CRC, budding cells of colorectal carcinoma displayed lack of membranous but highly increased cytoplasmic Ep-CAM staining and nuclear translocation of beta-catenin. They also demonstrate abnormal processing of Ep-CAM at the invasive margin of colorectal carcinomas. Their observations indicate that loss of membranous Ep-CAM is associated with nuclear beta-catenin localization and suggest that this contributes to reduced cell–cell adhesions, increased migratory potential, and tumor budding.

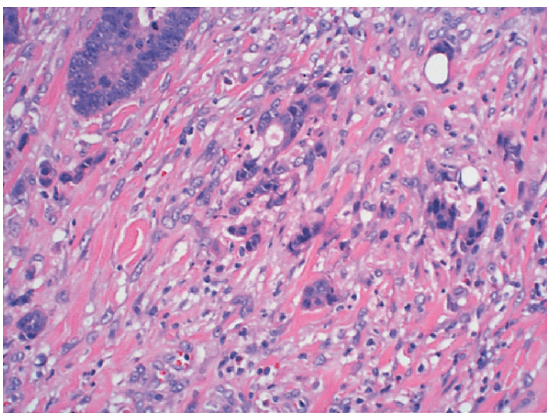

FIGURE 2.1. Tumor budding. Tumor budding, single cells or clusters composed of < 5 cancer cells, is observed at the invasive front of the tumor

IMMUNOHISTOCHEMISTRY FOR DIFFERENTIAL DIAGNOSIS

The accurate diagnosis of PDAC is very difficult because of its histological similarity to other poorly differentiated carcinomas, sarcomas or lymphomas. In addition, its rarity renders the differential diagnosis even more difficult. PDAC is pathologically associated with at least some gland formation or mucinous production (Hamilton and Aaltonen, 2000). Therefore, it is important in the diagnosis of PDAC that mucinous production is detected by the periodic acid-Schiff (PAS) procedure (Culling *et al*., 1977) or immunohistochemistry using

anti-MUC1 and MUC3 antibodies (Cao *et al*., 1997).

Immunohistochemical staining was performed as previously described (Shinji *et al*., 2006). In principle, paraffin-embedded tissue sections (3.5 µm) were immunostained using a Histofine Simple Stain PO (R) or (M) Max kit (Nichirei, Tokyo, Japan) as follows:

1. Immerse the slides in xylene. Remove after 10 min and shake off the excess xylene.
2. Repeat **Step 1** twice using fresh xylene.
3. Immerse slides in 100% ethanol. Remove after 3 min and shake off the excess 100% ethanol.
4. Repeat **Step 3** twice with fresh 100% ethanol.
5. Then, treat the slides with 90%, 80% and 70% ethanol in the same way as described earlier.
6. After immersion in water, immerse in phosphate buffer saline (PBS) for 5 min.
7. Immerse the slides in a 0.3% solution of hydrogen peroxide in absolute methanol for 30 min at room temperature.
8. Rinse them in PBS three times for 5 min each time.
9. Wipe areas around the sections on the slides.
10. Apply an appropriate antibody such as the rabbit polyclonal or the mouse monoclonal antibody in PBS containing 1% bovine serum albumin (BSA) to specimen slides.
11. Incubate them overnight at 4°C in a moist chamber.
12. Rinse the slides in PBS three times for 5 min each time.
13. Wipe areas around the sections on the slides.
14. Apply 2–3 drops of Simple Stain MAX PO (R) or (M) reagents to each slide to completely cover the sections on the slides. Incubate the slides at room temperature for 30 min in a moist chamber.
15. Rinse the slides in PBS three times for 5 min each time.
16. Wipe areas around the sections on the slides.
17. Apply 2–3 drops of diaminobenzidine-tetrahydrochloride (DAB) solution (20 µg of DAB powder and 100 µl of 5% H_2O_2 in 100 ml of Tris-HCl, pH6.5) to each slide to completely cover the sections. Incubate the slides at room temperature for 5–10 min, and observe them under a microscope.
18. Rinse the slides in distilled water for 5 min.
19. Immerse them in Mayer's hematoxylin for 2 min.
20. Wash them well in tap water.
21. Immerse them in PBS for 2–3 min.
22. Wash them in tap water.
23. Immerse in graded ethanol (70%, 80%, 90% and 100%).
24. Clear in xylene four times for 3 min each time.
25. Mount with mounting medium.

Negative controls were also prepared using the same procedure but without the primary antibody.

Several immunohistochemical markers have been reported for the differential diagnosis of CRC. CRC contains mostly low-molecular-weight cytokeratins (Chesa *et al*., 1986). In 90% of cases of well- and moderately differentiated adenocarcinomas of the colon, the immunohistochemical pattern shows negativity for cytokeratin 7 and positivity for cytokeratin 20, whereas the reverse pattern is rare (Chu *et al*., 2000; Kende *et al*., 2003). This information is very useful for the differentiation between primary CRCs and metastatic carcinomas

of other sites such as the lung, breast, and ovary. However, PDACs exhibit aberrant cytokeratin expression in 50% of cases; cytokeratin 7 positivity is increased in PDACs (Kende *et al*., 2003).

Villin has been reported as an immunohistochemical marker of CRC regardless of differentiation (Bacchi and Gown, 1991). Villin is a cytoskeletal protein associated with axial microfilament bundles of brush border microvilli, and is expressed in colorectal adenocarcinomas, but not in sarcomas, melanomas, or lymphomas. However, villin staining can be observed in gastric, pancreaticobiliary and ovarian adenocarcinomas, endometrioid carcinoma and renal cancer with distal tubular diffe--rentiation. Other markers for colorectal carcinomas such as CDX2 (Werling *et al*., 2003) and tumor-associated glycoprotein (Lottich *et al*., 1986) have been reported. Ultrastructurally, a feature of colorectal adenocarcinoma is the presence of both microfilaments entering the border and a mucin secretory product, but these cannot be used for definitive diagnosis (Lottich *et al*., 1986). The differential diagnosis for PDAC requires a combination of macroscopic and microscopic findings, and diagnosis of exclusion.

IMMUNOHISTOCHEMISTRY FOR MALIGNANT CHARACTERISTICS OF POORLY DIFFERENTIATED ADENOCARCINOMA

We have reported that the frequency of liver metastasis at the time of diagnosis is significantly higher in PDAC patients with neuroendocrine cell differentiation than in those without neuroendocrine cell differentiation (Shinji *et al*., 2006). Moreover, it was suggested that the microvessel density and VEGF-A expression level tend to be high in PDAC patients with neuroendocrine cell differentiation, and PDAC with neuroendocrine cell differentiation might induce liver metastasis through microvessel formation in the tumor as induced by VEGF-A. VEGF-A immunoreactivity was localized mostly in the cytoplasm of cancer cells and strongly localized in the cancer cells at the invasive front of the tumor. To differentiate between vascular and lymphatic endothelial cells in the microvessels of PDAC, immunohistochemistry using CD34 and D2-40 antibodies was performed. Vascular endothelial cells in colorectal cancer cell nests were positive for CD34 (Figure 2.2a and c, arrows), but not for D2-40 (Figure 2.2b and d, arrows). In contrast, lymphatic endothelial cells were negative for CD34 (Figure 2.2C, arrowhead), but were positive for D2-40 (Figure 2.2D, arrowheads). By using CD34 and D2-40 staining, the incidence rates of lymphatic and vascular invasion of PDAC with neuroendocrine cell differentiation were determined to be 89.6% and 77.1%, respectively.

Cancer cells with neuroendocrine differentiation have been observed in gastrointestinal carcinomas (Staren *et al*., 1990). Neuroendocrine cells possess a complete molecular machinery for the uptake and release of neurotransmitters and the secretion of neuropeptides. Some PDACs show neuroendocrine differentiation in some parts of the tumor; however, it is difficult to diagnose neuroendocrine differentiation in PDACs by routine hematoxylin and eosin (H&E) staining. Grabowski *et al*. (2001) reported that neuroendocrine differentiation

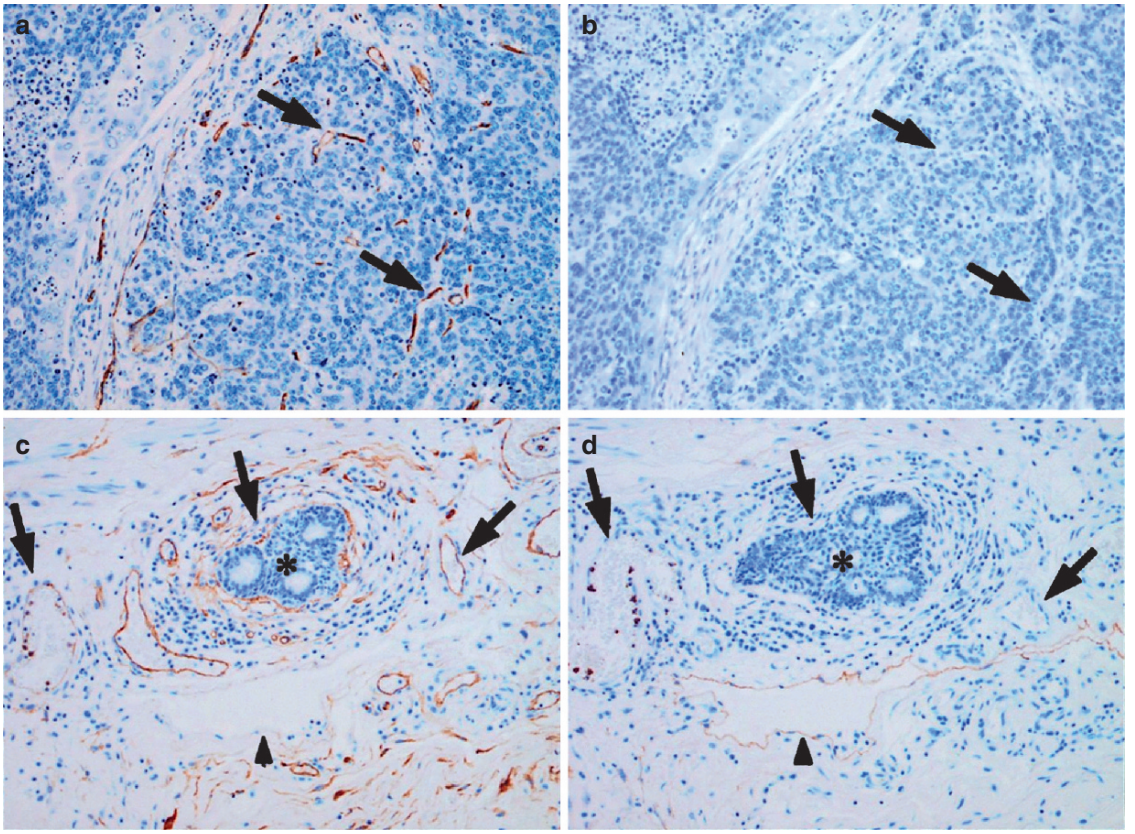

FIGURE 2.2. Immunohistochemical analyses for CD34 and D2-40 in PD adenocarcinoma with neuroendocrine cell differentiation. Tumor vascular endothelial cells were positively stained for CD34 (a and c, arrows), but were not positively stained for D2-40 (b and d, arrows). In contrast, lymphatic endothelial cells were not positively stained for CD34 (c, arrowhead), but these were positively stained for D2-40 (d, arrowhead). (Shinji *et al*., 2006.)

can be used as an independent prognostic factor in colorectal carcinoma of stages III and IV, and neuroendocrine differentiation was found more frequently among PDACs than in well- or moderately-differentiated adenocarcinoma. Furthermore, immunohistochemical studies of PDAC and undifferentiated colorectal adenocarcinomas have also shown neuroendocrine cell differentiation. Tumor cells with neuroendocrine differentiation exhibit characteristic neurosecretory granules in the cytoplasm as shown by electron microscopy. With immunohistochemistry, neuroendocrine cells are shown to be stained by neuroendocrine markers such as chromogranin A (Figure 2.3a, arrowheads), synaptophysin (Figure 2.3b), NSE, CD56, serotonin, vasoactive intestinal polypeptide (VIP), substance P, and somatostatin. Chromogranin A was observed in a matrix protein of large dense-core vesicles (100–400 nm in diameter) of neuroendocrine granules. Synaptophysin, an integral protein of the vesicle membrane staining membrane can be observed in small synaptic vesicle analogs (40–80 nm in diameter) by immunoelectron microscopical analysis (Sudhof

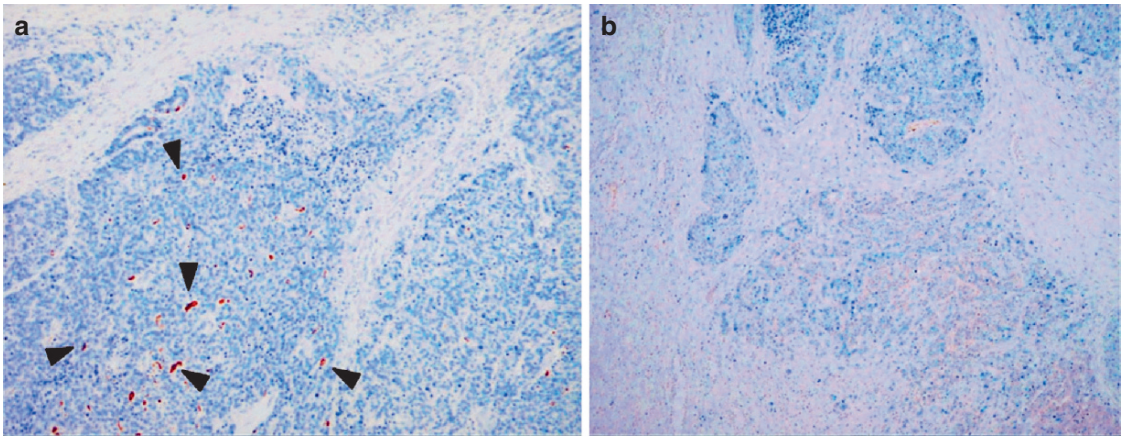

FIGURE 2.3. Immunohistochemical staining properties of chromogranin A and synaptophysin in PD adenocarcinoma with neuroendocrine cell differentiation Immunohistochemical staining of chromogranin A, (a arrowheads) and synaptophysin (b) show the cytoplasmic localization in PD adenocarcinoma cells with neuroendocrine cell differentiation. (Shinji *et al*., 2006.)

and Jahn, 1991). On the basis of immunohistochemical analysis, Staren *et al*. (1990) classified PDACs into four distinct groups, those composed entirely of exocrine or neuroendocrine cells, mixed exocrine–neuroendocrine cells, and predominantly exocrine carcinomas with an neuroendocrine cell subpopulation. Following these classification, the presence of neuroendocrine differentiation is associated with a poorer prognosis.

Moreover, lumican, a member of the small leucine-rich proteoglycan family that participates in the maintenance of tissue structure and tumor growth, is localized in the cytoplasm of PDAC cells with neuroendocrine feature and in some neuroendocrine cell carcinomas of the colon. Cytoplasmic lumican of neuroendocrine carcinoma cells might have a more inhibitory effect on cell growth than stromal lumican, although lumican expression in the stroma adjacent to the tumor was also observed (Shinji *et al*., 2005). Seya *et al*. (2006) reported that the survival rate of CRC patients with a high lumican expression level was significantly lower than that of CRC patients with a low lumican expression level.

IN SITU HYBRIDIZATION FOR MALIGNANT CHARACTERISTICS OF POORLY DIFFERENTIATED ADENOCARCINOMA

In situ hybridization for VEGF-A or lumican mRNA was performed using digoxigenin (DIG) nucleic acid detection kit (Roche, Diagnostic GmbH, Penzberg) as previously described (Shinji *et al*., 2006; Ishiwata, 2005). For the *in situ* hybridization of VEGF-A, a 200-bp *BamHI-EcoRI* cDNA fragment corresponding to nucleotide 1,183–1,368 of VEGF-A (NM_003376) and for lumican, a 215-bp *Bam*HI-*Eco*RI cDNA fragment, corresponding to nucleotides 663–858 of the human lumican cDNA sequence (U21128), were subcloned into the

pGEM-T vector and the authenticities were confirmed by sequencing (Ping Lu *et al*., 2002). The probes for VEGF-A mRNA and lumican mRNA were labeled with DIG-UTP using SP6 or T7 RNA polymerase from the DIG RNA labeling kit. The pretreatment of sections for *in situ* hybridization was performed as follows:

1. Immerse the slides in xylene. Remove after 10 min and shake off the excess xylene.
2. Repeat **Step 1** twice using fresh xylene.
3. Immerse slides in 100% ethanol. Remove after 3 min and shake off the excess 100% ethanol.
4. Repeat **Step 3** twice with fresh 100% ethanol.
5. Then, rehydrate the sections with 90%, 80% and 70% ethanol in diethyl pyrocarbonate (DEPC)-treated ultrapure water in the same way as described earlier.
6. Wash the slides twice with autoclaved PBS for 15 s each time, using a vibrator (Sakura Finetek Co. Ltd., Tokyo).
7. Incubate them in 0.2 M hydrochloric acid (HCl) for 20 min at room temperature.
8. Wash them in autoclaved PBS for 3 min.
9. Encircle the tissues on slides with PAP PEN (Daido Sangyo Co., Tokyo).
10. Apply 2–3 drops of 10–150 μg/ml proteinase K (Sigma, St Louis, MO) in PBS on the tissues encircled by PAP PEN, and then incubate for 15 min at 37°C in an OmniSlide Moist Chamber (A Thermo BioAnalysis Company, Teddington, UK).
11. Wash the slides with PBS for 5 min at room temperature using the vibrator.
12. Incubate them with 4% paraformaldehyde (PFA)/PBS for 5 min at room temperature.
13. Wash them with PBS for 5 min at room temperature using the vibrator.
14. Immerse the slides twice in 2 mg/ml glycine/PBS at room temperature for 15 min each time.
15. Wash them with PBS for 5 min at room temperature with vibrator.
16. Incubate the slides with 50% formamide/2X saline-sodium citrate (SSC) for 60 min at 42–55°C.

Then, the hybridization was performed as follows:

1. Mix 10–500 ng/ml of DIG labeled VEGF-A or lumican probe and hybridization buffer (0.6 M NaCl, 1 mM ethylenediamine tetra-acetic acid (EDTA), 10 mM Tris-HCl (pH 7.6), 0.25% SDS, 200 μg/ml t-RNA, 1X Denhardt's, 10% dextran sulfate, 40% formamide).
2. Denature the labeled probe with hybridization buffer for 10 min at 60°C and cool on ice.
3. Apply 100–150 μl of the denatured probe onto the slides and incubate overnight (O/N) at 42–55°C in the OmniSlide Moist Chamber.

Finally, the washes and detection of mRNA were performed as follows:

1. Wash the slides with 2X SSC for 20 min at 42–55°C in an OminiSlide Washing Module (A Thermo BioAnalysis Company, UK).
2. Wash them with 0.1–0.2X SSC for 20 min at 42–55°C in an OminiSlide Washing Module.
3. Incubate the slides with Buffer 1 (0.1 M Tris-HCl, 0.15 M NaCl, pH7.5) for 1 min at room temperature.
4. Incubate them with Buffer 2 (1% Blocking reagent in Buffer 1) for 60 min at room temperature.

5. Incubate them with Buffer 1 for 1 min at room temperature.
6. Incubate the slides for 30 min with the anti-DIG antibody diluted 1:2,000 in Buffer 1 containing 0.2% Tween 20 at room temperature.
7. Wash the slides twice with Buffer 1 containing 0.2% Tween 20 for 15 min each time at room temperature using the vibrator.
8. Incubate the slides with Buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 2 min at room temperature.
9. Prepare a color solution containing 10 ml of Buffer 3, 45 µl of nitroblue tetrazolium (NBT) solution and 35 µl of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP).
10. Cover the slides with 100–150 µl of the color solution and incubate them in a humidified chamber for 0.5–3 h in the dark.
11. Observe the slides under a microscope every 30 min and stop the color reaction by incubating the slides in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0).
12. Mount the sections with Mount-quick "Aqueous" mounting medium (Daido Sangyo Co., Ltd, Tokyo).
13. Observe the slides under light microscopy or light microscopy with a differential interference contrast (DIC) system.

By *in situ* hybridization, the expressions of VEGF-A mRNA and lumican mRNA in PDAC with neuroendocrine cell differentiation and in neuroendocrine cell carcinoma were detected as previously reported (Figure 2.4) (Shinji *et al*., 2006; Ishiwata, 2005).

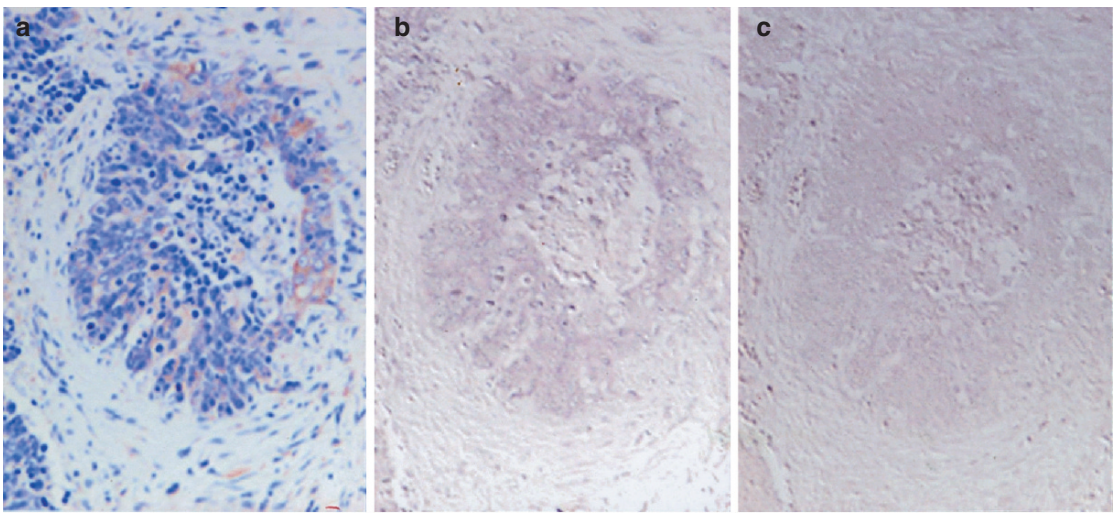

FIGURE 2.4. Immunohistochemical and *in situ* hybridization analysis for VEGF-A in PD adenocarcinoma with neuroendocrine cell differentiation. VEGF-A protein and VEGF-A mRNA were strongly expressed in PD adenocarcinoma cells with neuroendocrine cell differentiation (a and b, respectively). The sense probe analysis did not yield any positive signals in the PD adenocarcinoma cells (c)

THERAPY

During the past decade, significant progress has been made in the treatment of colorectal cancer because of advances in surgery, radiotherapy, and chemotherapy. The standard therapy for colorectal adenocarcinoma is surgical resection, and isolated distant metastasis to other organs can benefit from surgical resection (Abcarian, 1992). The benefit of preoperative or postoperative irradiation and/or chemotherapy for operative carcinoma of the colon has been reported in several centers (Wolpin *et al*., 2007). For patients with stage III colon cancer, an overall survival benefit associated with fluorouracil-based chemotherapy has been established. S-1, which contains tegafur, gimeracil and oteracil, based on a biochemical modulation of 5-fluorouracil, has recently been reported to be effective for patients with stage IV colon cancer (Ohtsu *et al*., 2000; Shirao *et al*., 2004). The orally administration of S-1 induced partial responses in 35–39.5% of patients (median survival time, 12 months) and the toxicity was manageable. The treatment of stage II disease is still somewhat less established, but may be appropriate against disease recurrence in the high-risk group (Wolpin *et al*., 2007).

It was reported that VEGF-A expression is significantly correlated with vascular invasion and lung metastasis, and tends to be correlated with liver metastasis in all PDAC patients. We previously reported that PDAC with neuroendocrine cell differentiation tends to be associated with a high VEGF-A expression level (Shinji *et al*., 2006). Neuroendocrine cell differentiation of PDAC in colorectal carcinoma was correlated with liver metastasis and tended to be associated with high MVD and VEGF-A expressions. Interestingly, three out of four patients who had VEGF-A-positive tumors with neuroendocrine cell differentiation had liver metastasis. It was considered that VEGF-A might play a partial role in the liver metastasis of PDAC with neuroendocrine cell differentiation. Recently, a human monoclonal antibody to VEGF-A (rhuMab VEGF-A), in combination with conventional chemotherapy, has been shown to increase the time for tumor development and even the survival rates of patients with colorectal cancer producing distant metastases (Ferrara *et al*., 2005).

Moreover, in the near future, the regulation for VEGF-A expression in PDAC patients with or without neuroendocrine cell differentiation might become a new molecular target for the inhibition of liver metastasis and tumor regression. However, in PDAC among CRCs, additional studies on the immunohistochemical staining of neuroendocrine markers might be necessary for evaluating the correlation between neuroendocrine cell differentiation and vascular invasion, and the effectiveness of anti-angiogenic therapy against the tumor.

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3
Colorectal Cancer: Immunohistochemical Diagnosis with Heterogeneous Nuclear Ribonucleoprotein K

Ayham Alnabulsi, Brian Carpenter, Colin Telfer, and Graeme I. Murray

INTRODUCTION

Colorectal cancer (CRC) ranks as one of the biggest killers in the developed world (Midgley and Kerr, 1999). It also has been estimated that at least half a million cases of colorectal cancer occur every year worldwide. Unfortunately, despite improvements in medical research and surgical provision, there has been little change in mortality rates from colorectal cancer during the past 40 years (Beart *et al*., 1995). The overall 5-year survival among CRC patients is only 40–50% (Jemal *et al*., 2006). Patients with CRC fall broadly into two groups at the time of presentation; the first group is diagnosed with late stage or metastatic disease and therefore has a very poor prognosis. The other group of patients is diagnosed with early stage disease and as a consequence has an overall good prognosis. Early detection of colorectal cancer correlates with high survival rates; for instance, 90% of patients that are diagnosed with Dukes' A tumors are alive 5 years after the initial diagnosis and those with Dukes' B stage have an 80–75% survival rate, compared with a steady decrease in 5 year survival in the later stages, down to 10% in patients with metastatic disease at presentation (Lloyd *et al*., 2006). The early stage group of CRC patients will only receive surgery but no chemotherapy as they are considered at low risk of recurrence of the disease. Regrettably, 30% of patients diagnosed with early stage CRC show a relapse of the disease.

Despite the fact that CRC develops slowly over a period of several years, it is proving very difficult to intervene clinically and identify patients who suffer from the disease at an early enough stage to permit complete recovery. The screening programs which do exist for CRC lack sensitivity and/or specificity for early detection. Also, there is a real limitation in the treatment process for CRC. The standard treatment options for CRC patients are surgery, radiation therapy (for rectal cancer), and chemotherapy; surgery remains the primary treatment of CRC. However, surgery alone is ineffective when CRC is diagnosed at the later stages and an adjuvant therapy is usually the likely course of treatment; chemotherapy and/or radiotherapy, depending on the patient's staging and overall medical condition, is recommended. Chemotherapy consists of a group of cytotoxic antineoplastic drugs

usually used to curtail metastasis or to shrink the tumor, with Fluorouracil (5-FU) the most commonly used therapeutic. However, there are problems with such treatments, most of today's cytotoxic drugs, including 5-FU, are relatively indiscriminate poisons, which target the machinery of cellular growth and division, resulting in systemic toxicities and undesired side effects.

To improve the current abysmal overall survival rates among CRC patients we must identify those affected with the disease at a very early stage, patients who are likely to experience disease recurrence after surgery, improve the process of diagnosis, prognosis, and modes of treatment for patients diagnosed with late stage or metastatic tumors. One promising tool with the potential to achieve all of the requirements mentioned above is the identification of molecular markers of colonic tumors. Tumor biomarkers are substances associated (overexpressed) with malignancy and can be detected in body fluids, circulating tumor cells in blood, lymph nodes, or bone marrow in the solid tumor. There are various clinical appltications for tumor markers, and several categories of markers can be defined. A diagnostic tumor marker is a marker that will aid in the detection of malignant disease in an individual. Ideally, a diagnostic marker should be tissue specific, not affected by benign diseases of that parti-cular tissue, and to exhibit high levels of sensitivity, especially if the marker is to be used for population screening purposes. A prognostic marker is a marker that assists clinicians in estimating the risk of disease recurrence and/or cancer-related death for an individual patient following the initial tumor resection, independent of future administration of adjuvant therapy (Schrohl *et al*., 2003). In contrast, a predictive tumor marker will envisage the patient's response or resistance to a given treatment. Predictive biomarkers hold the potential to be used to tailor design a patient's treatment through identifying responders from non-responders to a specific regimen. The tailor designing approach, termed theranostics, will prevent patients receiving ineffective treatments thus reducing the undesired side effects. Another category of tumor markers, termed monitoring markers, is used during follow-up of patients to supervise the efficacy of a specific therapy. Finally, a new and potentially important area includes the use of tumor markers for therapeutic application, as is the case of human epidermal growth factor receptor 2 (HER-2) oncogene which has become the focus of the Herceptin therapy for breast cancer (Hortobagyi, 2001). It is worth noting that the field of biomarker discovery is still in its infancy and so far only a handful of clinically useful biomarkers are available. This is the reason that at present there are significant efforts by drug companies, healthcare and regulatory authorities to bolster the speed of development and evaluation of new tumor markers. Currently, there is extensive academic and industrial activity in this area as it is forecasted that the biomarker market will quadruple to $21.2 billion in 2012 from $5.4 billion in 2005 (Business Insight, 2005).

BIOMARKERS

Tumor specific biomarkers can arise in a number of ways, such as a fusion or hybrid protein (e.g., an oncogene) that is reciprocally translocated and fused to an

active promoter of another gene, resulting in a hybrid which is constitutively active. The Philadelphia chromosome in chronic myeloid leukemia occurs through such a process, the abnormality occurs between chromosome 9 and chromosome 22. The translocation brings the proto-oncogene ABL on chromosome 9 to the middle of a gene designated BCR with an active promoter; thus, creating a new gene which when expressed drives cells to malignancy (Kurzrock *et al*., 1988). DNA sequences can be combined not only through translocations but also through inversions and insertions. Another kind of tumor biomarker is the oncofetal antigen that is normally expressed in cells during embryological development and subsequently repressed in adult tissues. Malignant cells have the tendency to switch on the expression of these genes making them very useful tumor targets. The most familiar and widespread oncofetal antigens are carcinoembryonic antigen (CEA) and α-fetoprotein (AFP). Common forms of tumor biomarkers arise from the overexpression of proteins in malignant cells. These markers will be expressed at normal levels by differentiating cells but are found at higher amounts in the corresponding tumor cells. The rise in biomarker levels can be detected in serum as is the case with prostate specific antigen (PSA) in prostate cancer.

Approved Biomarkers: Problems

Thus far there are only a limited number of tumor markers that are regularly used in clinical practice, being primarily used to help assess tumor response to treatment and to monitor tumor recurrence. In fact, the estrogen receptor α (ERα) is one of the few tumor markers routinely used clinically, the status of which is useful when deciding on adjuvant endocrine treatment in breast cancer patients (American Society of Clinical Oncology, 1998). A tumor biomarker that allowed early disease detection is prostate specific antigen (PSA). This antigen has been in widespread use for screening since the early 1990s when it achieved regulatory approval: a rise in PSA serum levels correlates with prostate cancer, Furthermore, the PSA level usually rises in the early stages of prostate cancers, facilitating early diagnosis. Concurrent with the widespread use of PSA screening, prostate cancer detection has increased, while mortality rates have decreased (McDavid *et al*., 2004). The advent of PSA screening has been marked by a decrease in the average age of diagnosis of prostate cancer among patients as well as a shift towards early stages of prostate carcinoma in the absence of symptoms: higher proportion of localized tumors. Despite the fact that PSA is among one of the best tumor biomarkers available for clinicians, it is by no means perfect; the value of PSA as a prostate specific biomarker is still being debated despite lower mortality rates (Hernandez and Thompson, 2004). The difficulty lies in the establishment of an optimal upper limit of normal value for PSA. Given that PSA can be increased in conditions such as benign prostatic hyperplasia and prostatitis, and conversely can be low in the presence of prostate cancer, it is difficult to determine a single value which indicates the presence of prostate cancer. Despite the controversy associated with it, PSA remains the only serum biomarker recommended by the American Cancer Society for use in the screening of malignancies. Most of the markers developed to date are

either not specific, or have 'high level of false-negatives' or 'high level of false-positives', making them unreliable, leading to expensive and unnecessary follow-up testing, or they are not elevated early enough in the disease process to facilitate cancer detection. These findings underline the need for more accurate biomarkers that can detect malignancies, distinguish benign from aggressive disease, and to identify those at risk of not responding to treatment.

Biomarkers of Colorectal Cancer

Carcinoembryonic antigen (CEA) and carbohydrate antigen (CA19-9) fall into the category of monitoring markers, and are the two most common tumor markers for performing clinicopathologic investigations on colorectal carcinomas. These two markers are oncofetal antigens, expressed in several different cancers, but especially carcinomas of the gastrointestinal tract. Carcinoembryonic antigen is a glycoprotein that plays a vital role in biological processes such as adhesion and apoptosis of the tumor cells (Hammarstrom, 1999), and is a useful marker to determine tumor recurrence (Duffy *et al*., 2001). The presence of CEA is one of the most frequently used tests in follow-up, after CRC surgery, and a large number of studies have been published since the 1970s highlighting the crucial role CEA measurements play in the management of these patients. Unfortunately, a number of conflicting studies have questioned the effectiveness of the CEA test in follow-up after CRC resection (Koerner *et al*., 2006). The value of CEA as a prognostic marker is currently in doubt. Some studies have shown that CEA is an independent prognostic factor; however, in additional studies this correlation failed to emerge (Kos *et al*., 1998). Interestingly, as a diagnostic tool, CEA proved to be an inadequate test and in a report published by American Society of Clinical Oncology, an elevated CEA has an unacceptably low positive predictive value, with excessive false-positives. Also, because elevated CEA occurs in the advanced stage of incurable cancer but is low in the early curable disease, the likelihood of a positive result affecting a patient's survival is diminished. Another pitfall of CEA marker is the fact that CEA is often positive in malignancies other than colonic, such as in cancers of the breast, lung, pancreas, stomach, and ovary. Although CA19-9 has been reported to be less sensitive to detect colorectal carcinoma than CEA, it still proved a useful biomarker for assessing tumor recurrence and overall prognosis. Simultaneous use of the two markers (CEA and CA19-9) has found use in evaluating the therapeutic effect and monitoring the recurrence of advanced colorectal cancer. It is worth noting that CA19-9 serum concentration also serves as an early indicator of response to chemotherapy in advanced pancreatic cancer (Ziske *et al*., 2003).

Another CRC diagnostic biomarker which has been recently approved by the Food and Drug Administration is UDP-glucuronosyltransferase (UGT1A1). It was found that genetic polymorphism in UGT1A1 was predictive of severe toxicity observed in a group of patients receiving irinotecan treatment in cancer chemotherapy (Ando *et al*., 2000). Irinotecan is used for the treatment of metastatic colorectal cancer; the drug acts by inhibiting topoisomerase I. Irinotecan is a prodrug metabolized to its active metabolite SN-38 that is further conjugated by hepatic UGT1A1 to yield the more

polar and inactive metabolite. A genetic polymorhpism within the promoter of the human UGT1A1 gene (UGT1A1*28) is found to influence a patient's ability to break down irinotecan, which can lead to increased blood levels of the drug and a higher risk of side effects including diarrhoea and neutropenia (Ando *et al*., 2000). Hence, the required need for UGT1A1 screening prior to irinotecan administration to CRC patients with the polymorphism.

Various putative molecular markers have been extensively investigated with respect to prognosis and response to therapy in CRC patients, albeit these markers are yet to be approved for clinical utility. For instance, microsatellite instability (MSI) is seen in as many as 15–20% of patients with sporadic CRC, and is a prognostic marker of an overall improved survival among patients (Ishimaru *et al*., 1995). On the other hand, a deletion of chromosome 18q, which harbors the candidate tumor suppressor genes DCC, SMAD2, and SMAD4, in CRC patients is associated with poor clinical prognosis. A number of studies found that patients with Dukes' B cancers and 18q allelic loss had a prognosis similar to that of patients with Dukes' C (Lanza *et al*., 1998). In addition, mutations and overexpression of p53 are associated with poor outcomes in CRC (Bell *et al*., 1993). Thymidylate synthase is an enzyme active in DNA synthesis that is targeted by 5-FU and similar chemotherapeutic agents. Overexpression of this enzyme is associated with a poor prognosis but also with improved sensitivity to 5-FU-based chemotherapy (Edler *et al*., 2002).

Demand for Colorectal Biomarkers

Despite advances in understanding the biology and natural history of CRC, survival rate has not improved significantly in recent years. Apart from timely surgery, few therapies are effective. The low survival can be assigned to the fact that there are only a handful of biomarkers available for curing CRC patients. Most of the markers are not specific or sensitive enough to identify CRC at an early stage or aid more effectively with diagnosis, prognosis, and mode of treatment. Patients have to be subjected to a number of screening programs to make the initial diagnosis. A common inexpensive investigation used is the fecal occult blood test (FOBT) to detect traces of blood in the stool. Although FOBT has achieved some success, it is not without its problems. This test is based on the assumption that the colorectal tumor releases small amounts of blood continuously; however, in reality this process occurs sporadically, hence the sensitivity of a single test may be as low as 30–50% (Mandel *et al*., 1993). Furthermore, the presence of blood in stools is also indicative of other diseases, for instance, peptic ulcers resulting in a large number of false-positives. In spite of its limitations, FOBT is now in routine use as a screening tool in the United Kingdom. Other assays, such as colonoscopy and sigmoidoscopy, even though more accurate than FOBT, are still avoided by patients because they are invasive and uncomfortable. Additionally, such tests have low sensitivity when detecting small and flat adenomas (Rex *et al*., 1997; Rembacken *et al*., 2000) not to mention potential for severe complication such as colonic perforation and severe bleeding (Liebeman *et al*., 2000).

In brief, there is a genuine deficiency of available sensitive and specific screening tests for CRC. On the one hand, the few screening assays that do exist are always likely to yield false-positives and false-negatives

as well as being invasive and uncomfortable. On the other hand, the tumor biomarkers that currently exist are also unreliable and in some instances their application for diagnosis and screening has been questioned. It is clear that a more robust set of tumor markers are required to improve the abysmal survival rates among patients with colorectal cancer. Therefore, a strong rational focused on overcoming the inadequate technologies currently available for tumor biomarker discovery need to be designed and implemented.

COLORECTAL BIOMARKER HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN K

Through combining a variety of discovery and validation technologies heterogeneous nuclear ribonucleoprotein K (hnRNPK) was identified as a novel CRC biomarker that holds the potential to facilitate both diagnosis and prognosis. The following section provides a short background introduction to hnRNPK followed by a description of each of the methodologies employed. HnRNPK is a member of heterogeneous ribonucleoprotein (hnRNP) family. There are at least 20 hnRNP members playing wide range of functions including mRNA stabilization and regulation, mRNA splicing, mRNA nuclear-cytoplamic shuttling, translational activation, translational silencing, transcriptional control, and as structural components of important DNA-protein complexes (Carpenter *et al.*, 2006a). Owing to various functions played by hnRNPs, it is of no surprise to discover their involvement in tumor development (Carpenter *et al.*, 2006a). HnRNPK has emerged recently as a critical protein in cancer progression; it has been confirmed that hnRNPK binds poly C on DNA sequences, with such an element being present in the oncogene c-MYC. Interestingly, it was reported that overexpression of hnRNPK increased the transcriptional activity of a c-MYC reporter gene (Carpenter *et al.*, 2006b) and also modulates the oncogene *c-SRC* expression (Ritchie *et al.*, 2003). HnRNPK is overexpressed in SV-40 transformed human keratinocytes and in human breast cancers. Based on the published studies it is apparent that a deregulated hnRNPK may be associated with pathological conditions such as malignancies.

Methodology

The method employed in this study for the discovery and validation of colonic tumor markers are summarized in Figure 3.1. Briefly, for the discovery process, two-dimensional gel electrophoresis (2DGE) proteomics was applied, and for the validation step, both semi-quantitative RT-PCR and immunohistochemistry (IHC) were used. The discovery and validation steps were performed on samples from a CRC database containing both tumor and normal associated tumor samples. The colorectal tissue samples used were obtained from a high value human tissue bank into which had been collected tumor and pathologically normal (disease-free) tissues from individual patients after resection. It was essential to collaborate and coordinate with the surgeons, pathologists and laboratory staff to ensure that tissues were preserved as rapidly as possible. All tissues were dissected and advanced to processing (snap freezing or formalin fixation) within 30 min of surgical removal. Tumor samples were selected from viable (non-necrotic) regions of the tumor, whilst normal

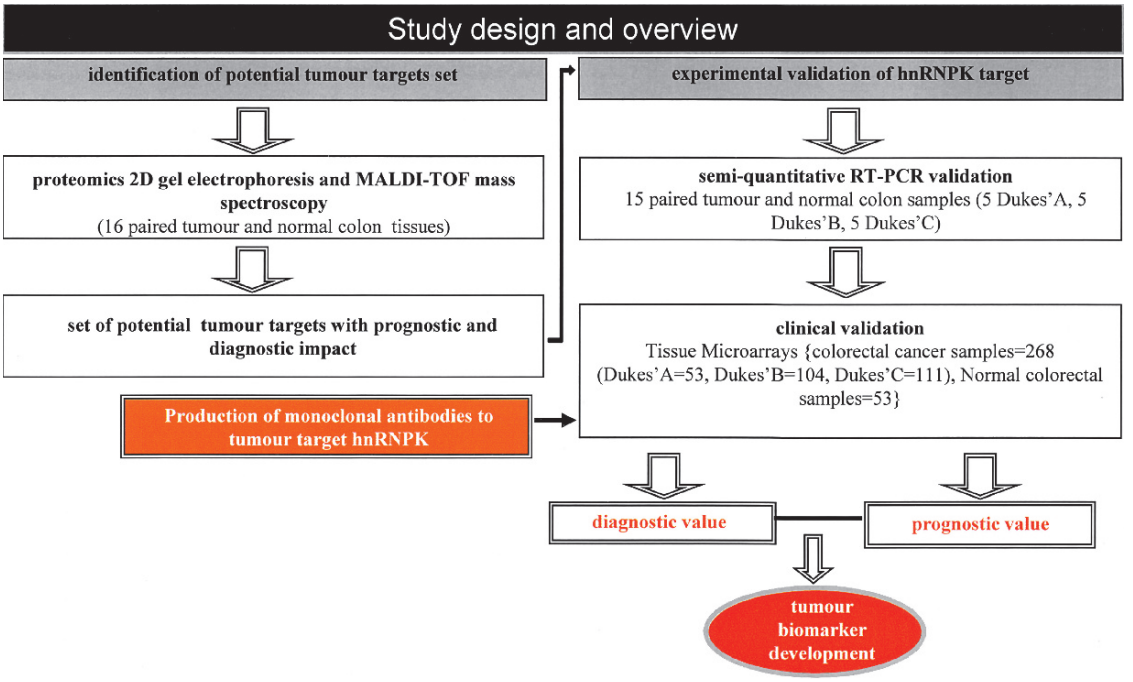

FIGURE 3.1. Study design and an overview of a biomarker discovery platform. The method involves utilizing current technologies for biomarker discovery. 2DGE and MALDI-TOF mass spectroscopy were employed initially to identify potential biomarkers. The potential biomarker was then subjected to two validation steps, starting with semi-quantitative RT-PCR. Once verified the potential biomarker was taken forward and subjected to clinical validation by applying immunohistochemistry using a tissue microarray block of colorectal cancer. All experiments were performed on a collection of colorectal sample pairs (normal and tumor) acquired from a high quality tissue biobank

colon mucosa samples were obtained at a distance of at least 5 cm (often 10 cm) from the tumor sample and were examined by an expert pathologist (Professor Graeme Murray) to confirm the diagnosis. For each case recruited, there were both formalin-fixed paraffin-embedded tissue blocks and paired frozen samples of tumor and normal tissues (snap-frozen in liquid nitrogen and stored at −80°C). Timely preservation guarantees that tissue quality is not compromised which is very important for subsequent analysis.

2D GEL Electrophoresis (2DGE)

The application of proteomics to identify biomarkers has become a major new paradigm (Ornstein and Tyson, 2006). To date, most molecular profiling studies of cancer have focused on mRNA transcript analysis. However, there are distinct advantages of proteomic studies because proteins are ultimately responsible for the disease phenotype. Proteomics can identify alterations in post-translational modifications, cellular trafficking, and even total genome expression. There remain, however, a number of fundamental challenges to the cost-effective implementation of biomarker discovery and validation. At the heart of the challenge is the sheer complexity of the human proteome. Due to alternative splicing and post-translational modification processes the human proteome

is estimated to contain 1,000,000 functionally distinct proteins. In any given cell a subset of the genes is expressed and subsequently the number of different proteins will be reduced, though still numbering in the 10,000s. Typically 2DGE will reveal ~ 1,500–2,000 proteins; thus, not detecting proteins expressed at low levels. Other major limitations of 2DGE are that it is labor-intensive and the gel does not resolve highly basic proteins or those smaller than 10 kDa. However, because most clinically useful biomarkers are high-abundant large proteins, 2DGE is still an ideal technology for cancer biomarker discovery studies. The most important and often least addressed piece, in biomarker discovery is the availability of highly standardized and high quality materials for analysis, principally as proteins are very sensitive to degradation. Similarly, the nature of sample collection and processing can greatly affect protein recovery and lead to spurious identification of differential protein expression. It has become apparent that the majority of samples held in historical tissue banks are of little value for proteomics as in many occasions the tissue specimens were not collected, stored, and transported under standardized protocols. Because of that most studies must look to prospective sample collection under tightly regulated collection, handling, and analytical protocols.

The rationale behind 2DGE is straight forward; for the first dimension, proteins are separated according to their isoelectrical focusing point: a gradient of pH is applied to a gel and an electric potential is applied across the gel, making one end more positive than the other. At all pHs other than their isoelectric point, proteins will be charged. If they are positively charged, they will be pulled towards the more negative end of the gel and if they are negatively charged they will be pulled to the more positive end of the gel. The proteins applied in the first dimension will move along the gel and will accumulate at their isolelectric point. That is, the point at which the overall charge on the protein is zero (i.e., a neutral charge). Subsequently, proteins are separated in the second dimension based on the molecular weight. To visualize a protein fingerprint for CRC by 2DGE, frozen sections (10 μm in thickness) of tumor and normal tissue were cut using a cryostat and then sections of adenocarcinoma tissue samples and sections of patient-matched normal colorectal mucosa samples were solubilized in a detergent lysis buffer. Equal protein amounts of normal and tumor samples were loaded onto Immobilon strips, pI 3–10 (1st dimension), focused, and subsequently resolved by SDS-PAGE (second dimension). It is worth noting that prefractionating (using lysis and centrifugation to separate cytoplasmic from nuclear and cell membrane fractions) the proteins prior to 2DGE separation reduced the complexity of the protein mixture, and hence circumvented a major drawback of 2DGE technology. Following completion of electrophoresis, gels were stained with Coomassie Blue to visualize protein spots. To ensure reproducibility and to eliminate one of the anomalies, 2DGE analysis was performed on 16 matched pairs of frozen tumor and disease-free normal colorectal tissues samples.

Spots identified as being expressed specifically in the cancer tissues were excised from the gel and subsequently identified through peptide mass fingerprinting. In summary form, the unknown protein of interest is cleaved into peptides by a protease

such as trypsin. The collection of peptides resulting from this cleavage comprises a unique identifier of the unknown protein. The absolute masses of the (still unknown) peptides are accurately measured with a mass spectrometer. These known masses are then *in silico* compared to the genome. Computer programs translate the known genome of the organism into proteins, then theoretically cleave the proteins into peptides with the same protease (for example trypsin), and calculate the absolute masses of the peptides from each protein. They then compare the masses of the peptides of the unknown protein to the theoretical peptide masses of each protein encoded in the genome. The results are statistically analyzed to find the best match between the unknown protein and the computer generated fragments arising from the known protein.

Semiquantitative Reverse Transcription-Polymerase Chain Reaction

To build upon our initial finding that hnRNPK was overexpressed in CRC tissues we sought additional validation through the use of semiquantitative reverse transcription polymerase chain reaction (RT-PCR). This technique provides a cost- and time-effective way of validating target biomarkers, if used in the early stages of biomarker discovery, and gives an indication of the abundance of targets at the transcriptome level. RT-PCR is a multiple-step polymerase chain reaction (PCR) accomplished by isolating total RNA from cells, using reverse transcriptase to create a pool of complementary DNA (cDNA), followed by amplification of a fragment of cDNA target through the use of two specific oligonucleotide primers that flank the DNA target and multiple rounds of thermocycling (Figure 3.2). The amount of a specific gene transcript can theoretically be measured by estimating the intensity of the amplified DNA band on an ethidium-bromide-stained agarose gel which corresponds to the abundance of that template.

In this study the transcriptional expression of 15 pairs of colorectal samples (five Dukes' A, five Dukes' B, and five Dukes' C) with their normal counterparts were assessed using semiquantitative RT-PCR. Fresh frozen tissue samples obtained from primary colorectal cancer resections, were lysed to isolate total RNA. Purity was determined by the ratio of OD at 260 nm/OD at 280 nm and purity values between 1.8–2.3 were considered for further analysis; a 260/280 nm ratio lower than this is indicative of protein contamination. The integrity of RNA preparations were also qualitatively assessed by denaturing agarose gel electrophoreses. A denaturing gel system is employed because most RNAs form extensive secondary structure via intramolecular base pairing, and this prevents it from migrating strictly according to its size. Ribosomal bands 18S and 28S are used as good quality controls for RNA integrity; sharp ribosomal RNA bands without a leading smear suggest a high quality RNA. Total RNA was used for first strand cDNA synthesis. Specific hnRNPK primers were designed and used for PCR analysis, and PCR products were resolved by agarose gel electrophoresis. The gel was then photographed under transillumination using bioimaging machine; densitometric analysis of images from RT-PCR gels was performed using bioimaging analysis software. The amount of DNA present in a single band was quantified and the value was divided by the corresponding value of the positive control RPS13 (ribosomal

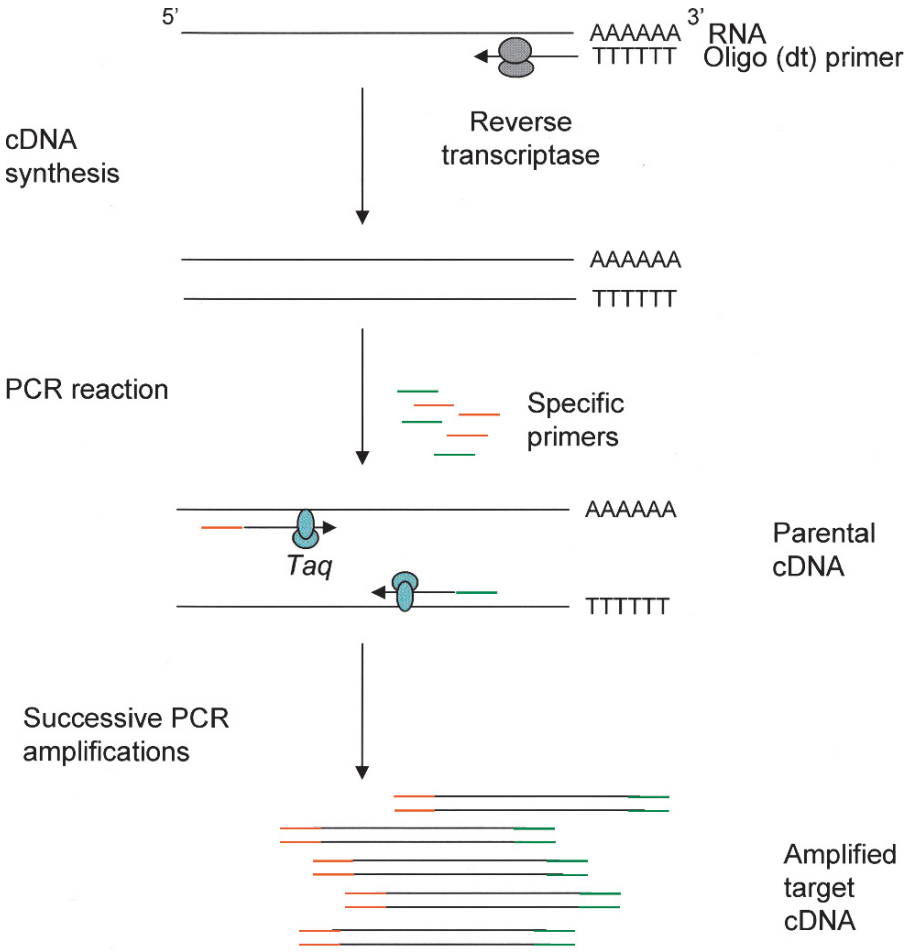

FIGURE 3.2. A simplified diagram of RT-PCR molecular technique. Total RNA is isolated from cells and then reverse transcribed into its cDNA by the action of reverse transcriptase, followed by amplification of the resulting cDNA using PCR. PCR amplification of a cDNA target is accomplished through the use of two specific oligonucleotide primers that flank the DNA target, the action of DNA polymerase (*Taq*) and rounds of thermocycling

protein S13) to normalize the final reading. RT-PCR is based on the dogma that the level of mRNA in cells is reflective of the functional protein abundance. However, major drawbacks to RT-PCR are that many proteins do not conform to the paradigm that the levels of mRNAs correspond to the abundance of functional protein. Also, another drawback is its lack of data regarding protein localization. Despite these major pitfalls, RT-PCR is still a popular technique for validation of biomarkers especially if complemented by more informative methodologies such as immunohistochemistry.

Monoclonal Antibody to Heterogeneous Nuclear Ribonucleoprotein K

We employed immunohistochemistry to validate hnRNPK as a CRC biomarker. As there was a lack of high quality antibodies for hnRNPK, we designed and produced

a monoclonal antibody to hnRNPK. We raised monoclonal antibodies against a synthetic peptide corresponding to a short fragment of hnRNPK. One of the main advantages of using synthetic peptides is that specific epitopes can be targeted. A BLAST search was performed and a sequence with minimal homology was selected to reduce the chance of nonspecific antibody binding. Moreover, features including length, hydrophobicity, series of specific amino acids, and antigenicity were considered. A monoclonal antibody for hnRNPK was produced using the standard protocol. Briefly, a ten amino acid C-terminal peptide to hnRNPK (SVKQYYSGKFF) conjugated to ovalbumin was used to immunize mice three times over a period of 5 weeks, followed by a booster. Screening of test bleeds against the immunizing antigen using ELISA enabled the identification of animals with the highest antibody titres (indicative of immune response to hnRNPK protein fragment). The spleen cells of these animals were fused with myeloma cells. Through the fusion, spleen cells become immortalized which facilitates growth in tissue culture; such cells secrete the antibody in the growth media. However, as the original spleen cells contain a mixed population, it was necessary to isolate a single clone cell which synthesizes only the antibody of interest. Again, such clones can be evaluated by ELISA. To further validate the specificity of the antibody, Western blotting using the conditioned supernatant in which the immortalized clone had been grown in was employed.

Immunohistochemistry (IHC)

Immunohistochemistry is a technique that plays, and will undoubtedly continue to play a prominent role in the field of biomarker validation. It is capable of providing detailed information regarding target localization, expression across the different stages of the disease, and correlation with clinical survival. A human CRC tumor tissue microarray (TMA) was constructed, which consists of paraffin blocks in which up to 1,000 separate tissue cores are assembled in an array fashion to allow simultaneous histological analysis. The technique of TMA was developed to address the major limitations in molecular clinical analysis of tissues including the cumbersome nature of procedures, limited availability of reagents and tissue resources, and to facilitate the automation and acceleration of tumor marker discovery by immunohistochemical analysis. In the tissue microarray technique, a hollow needle is used to remove tissue cores from regions of interest in paraffin-embedded blocks such as tumor samples. These tissue cores are then inserted in a recipient paraffin block in a precisely spaced, array pattern. Sections from this block are cut using a microtome, mounted on a microscope slide and then analyzed by immunhistochemistry (Figure 3.3). Each microarray block can be cut into 100–500 sections, which can be subjected to independent tests. The TMAs are of sufficient size to permit rapid validation of candidate colorectal tumor antigens and the generation of data of high statistical significance.

Immunhistochemistry for hnRNPK was carried out; the tissue was dewaxed using xylene, and rehydrated before an antigen retrieval step (citrate buffer) was performed by microwaving. Through the fixation of tissues (usually through the use of formalin), the antigen (in this case hnRNPK) can often been masked through protein–protein

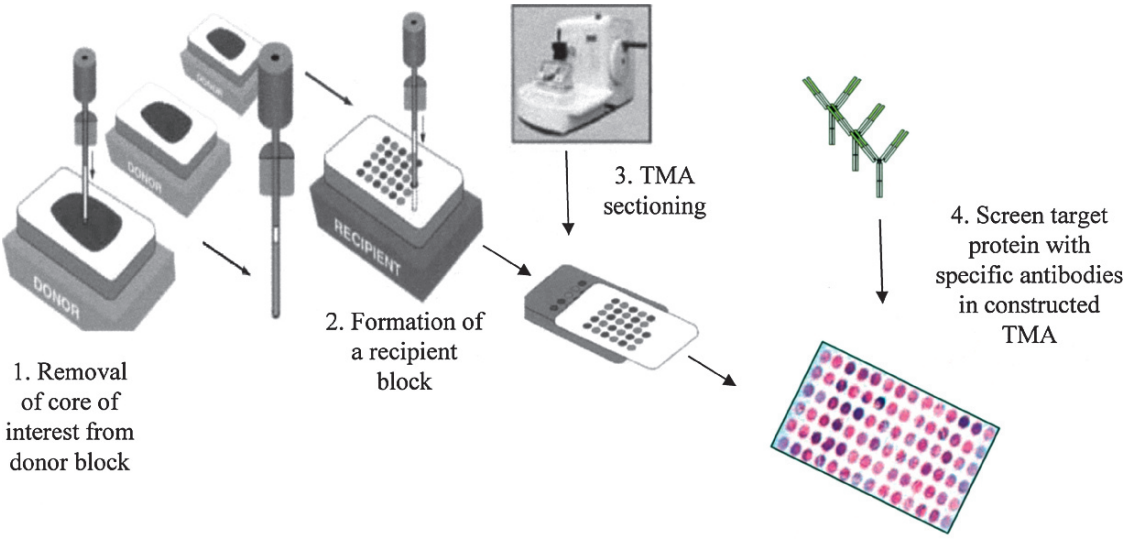

FIGURE 3.3. Tissue microarray construction. Tissue microarray consists of paraffin embedded tissue cores that are acquired from tissue donor blocks from surgical pathology. The tissue cores are inserted into a paraffin block using a specially designed instrument. Tissues are inserted at high density in a single paraffin block, termed the recipient block. Sections from this block are then cut with a microtome and prepared for immunohistochemical analysis by screening the target protein with specific antibodies. Intensity of immunoreactivity is scored semi-quantitatively by observing the tissue microarray slide onto a microscope

interactions. It is thought that through antigen retrieval these masked domains become uncovered which facilitates antibody binding. The primary hnRNPK antibody was applied to the sections and incubated for 1 h followed by washing and addition of the secondary antibody (1 h). After washing, antibody staining was visualized using diaminobenzamidine. The intensity of immunostaining in each section was assessed by light microscopic examination by two independent observers. The scoring method applied for hnRNPK was as follows: the intensity of immunoreactivity in each section was graded as negative = 0, weak = 1, moderate = 2 and strong = 3. The proportion of cells staining positively were assessed as no cells = 0, 1–25% of cells = 1, 26–50% = 2, 51–75% = 3 and 76–100% = 4. Furthermore, staining of the cellular compartment of nucleus and cytoplasm was also noted. To analyze the mean total, nuclear and cytoplasmic hnRNPK scores were added to give a range of scores from 0 to 6.

RESULTS

Identification of Overexpressed hnRNPK in Colorectal Cancer

2DEG-proteomics was used as the method of choice to identify new tumor targets. Proteomic analysis was performed in duplicate on 16 matched pairs of tumor and normal colorectal tissues. Spots which were exclusive to the tumor samples were subsequently excised and identified by MALDI-TOF. One protein which could be detected in 88% of CRC tumors (14/16) was found to be consistently elevated in

tumor samples compared to normal. When semi-quantitative RT-PCR was employed to verify 2DEG findings, similar results were observed (Figure 3.4). RT-PCR results showed a considerable increase in hnRNPK levels in malignant colon samples in comparison with normal ones; thus verifying findings in the proteomics experiment. As can be seen in Figure 3.4, hnRNPK expression was elevated in 93% (14/15) tumor samples compared to normal colon samples. Also, Figure 3.4 shows hnRNPK to be expressed at high levels across the different Dukes stages examined. Finally, and in agreement with the proteomics analysis and RT-PCR results, hnRNPK staining visualized using immunohistochemistry was significantly higher in primary tumor compared to normal colon ($p < 0.001$) (Figure 3.5).

Localization of hnRNPK in Colorectal Cancer

One intriguing finding detected by immunohistochemistry and not proteomics or RT-PCR was the aberrant localization of hnRNPK in colorectal cancer (Figure 3.5). In normal colon, hnRNPK localization was exclusively present in the nuclei of crypt epithelial cells with no cytoplasmic expression. On the other hand, strong cytoplamic hnRNPK presence was detected in primary colorectal tumors. Analogous hnRNPK staining profile observed in primary tumors was also detected in the lymph

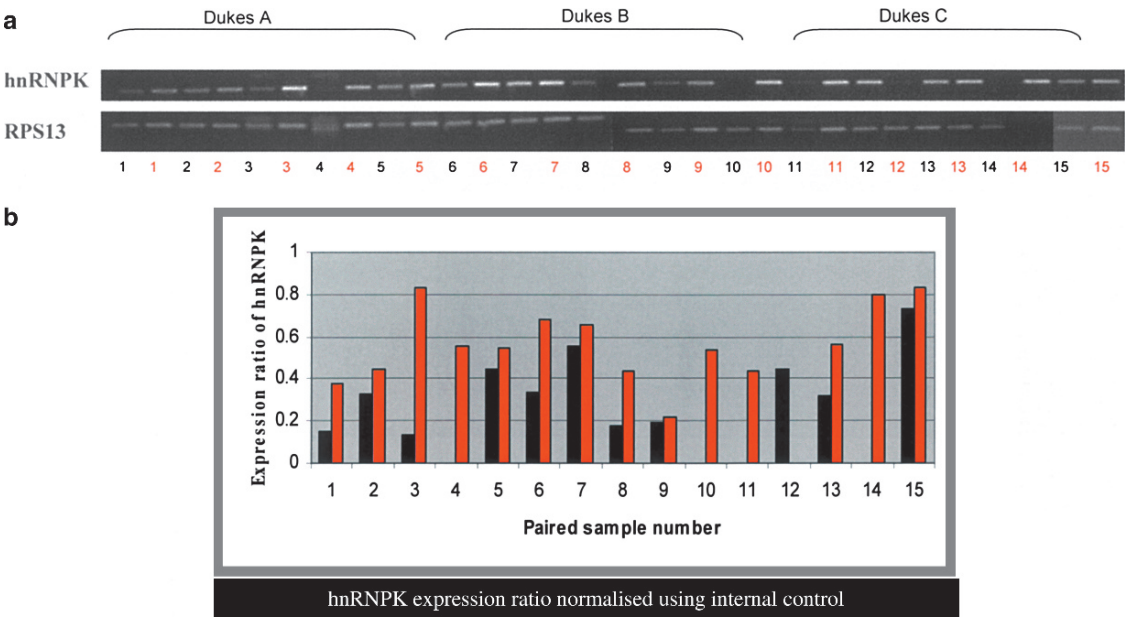

FIGURE 3.4. HnRNPK expression in human colorectal tumors of different stage. The expression of hnRNPK was evaluated by semi-quantitative RT-PCR and compared with that of the 40S ribosomal protein S13 protein (RPS13) gene, shown to be expressed at nearly identical levels in normal and neoplastic colorectal tissues. (a) Gel of RT-PCR products from 15 paired colorectal epithelium of normal versus tumor. Lane M, molecular size markers. (b) Normalized results are expressed as the ratio between hnRNPK and RPS13 expression

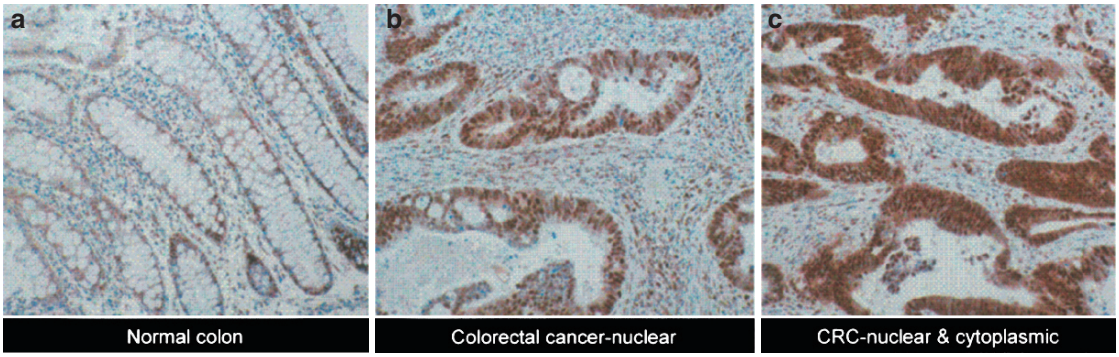

FIGURE 3.5. HnRNP K immunostaining in normal colon versus colorectal cancer. (a) In normal colon tissues, hnRNPK immunoreactivity is exclusively present in the nuclei of the crypt epithelial cells. (b) Strong nuclear and weak cytoplamic staining in primary colorectal cancer. (c) Strong nuclear and cytoplamic immunostaining in primary colorectal cancer. (Reproduced with permission from Carpenter *et al*., 2006b.)

node metastatic samples. Furthermore, a decrease in nuclear hnRNPK levels can be observed when comparing normal colon with primary tumor and primary tumor to lymph node metastasis. Interestingly, this decrease in hnRNPK staining intensity was found to be significant ($p < 0.001$ and $p = 0.006$). The ability of immunhistochemistry to confer data regarding the localization profile of target proteins highlights one of the main advantages of employing this technology in the process of biomarker validation.

Relationship of hnRNPK Levels, Clinicopathological Parameters, and Overall Survival

HnRNPK immunoreactivity scores were also noted in different stages of colorectal cancer. Such analysis generally confers very valuable data regarding the correlation between the expression level of the marker and tumor stages; hence, underlying the diagnostic and prognostic potential of the biomarker examined. Interestingly, we found a significant increase in both nuclear and cytoplasmic hnRNPK staining as colorectal cancer progressed from Dukes' A to Dukes' B to Dukes' C Tumors. Highest expression of nuclear and cytoplasmic hnRNPK was detected in Dukes' C and such increase was significant when compared to hnRNPK levels in Dukes' B ($p = 0.007$ and $p < 0.001$, respectively). No correlation was found between nuclear hnRNPK or cytoplamic hnRNPK and tumor site, tumor differentiation, and gender or age.

Kaplan-Meier survival analysis showed no relationship between the overall survival rate in patients with CRC and the expression levels of nuclear, cytoplasmic or total hnRNPK expression. However, in those patients diagnosed with Dukes' C tumors whose tumors had low or negative nuclear hnRNPK score, there was a poorer prognosis compared with Dukes' C patients whose tumors had high nuclear hnRNPK score ($p = 0.0093$, Figure 3.6). The mean survival time was 23.4 months for the poor survival cohort ($n = 15$), whereas in the good survival cohort ($n = 87$) the mean survival was 64.1 months. There

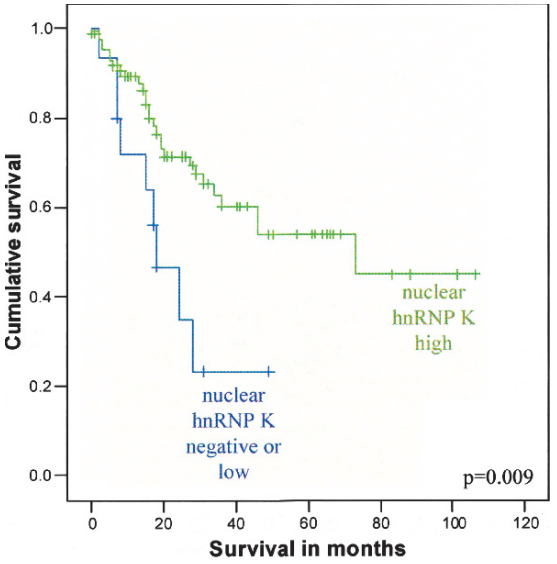

FIGURE 3.6. Kaplan Maier survival analysis comparison in patients whose tumors have a high nuclear hnRNPK expression and patients whose tumors have moderate or low hnRNPK nuclear levels. Significantly there is poorer survival in colorectal cancer patients whose tumors showed moderate or low nuclear hnRNPK levels ($p = 0.009$). (Reproduced with permission from Carpenter *et al.*, 2006b.)

was no relationship between either nuclear or cytoplamic scores of hnRNPK across the colorectal cancer stages examined and overall survival.

DISCUSSION

Colorectal cancer is one of the most common cancers in industrialized countries and the affluent world, with very high mortality rates. The incidence rates with colorectal cancer are estimated to rise even further as the average age of the population increases. While age is considered the most important factor in the etiology of colorectal cancer, sedentary life style, low fiber diets, fat rich diets and meat are among a number of factors associated with the increased risk. The disease has reached epidemic proportions in the United States and Japan and moreover, the incidence of the disease and the prevalence rates are likely to increase without diagnostic and therapeutic advances. As many as 50% of patients will be diagnosed with the disease at Dukes' C who will have 5-year survival of 60–30%. On the other hand, after colon cancer resection, patients with Dukes' A disease have a 5-year survival of 90%, while patients with Dukes' B disease have a 5-year survival of ~ 80–75%. The standard treatment options for CRC patients are surgery, radiation therapy, and chemotherapy. Surgery remains the primary treatment of CRC, while chemotherapy and/or radiotherapy, depending on the patient's staging and overall medical condition, may be recommended. Chemotherapy is usually used to curtail metastasis or to shrink the tumor, with 5-FU the most commonly used therapeutic. Even with the treatment, 40–50% of patients ultimately relapse and die due to metastasis of the disease. These figures highlight how imperative it is to detect the disease at its earliest stages to improve dramatically the patient's prognosis. Owing to the low survival rates among patients with CRC, there is a demand for tumor biomarkers to facilitate early diagnosis, prognosis, and tailor design a patient's therapy regimen.

To meet this demand we adopted an approach to bring together a variety of technologies to form a biomarker discovery and validation platform. This platform was enriched through having access to a human CRC database with matching clinicopathological data. We opted to choose 2DGE proteomics as an initial step in the biomarker discovery process. In spite of a number of fundamental limitations such

as the low resolution power of low abundance and small basic proteins, 2DGE still represents a highly competent technology for cancer biomarker discovery, as thus far it has been shown that the most clinically useful biomarkers are high-abundant large proteins. Potential biomarkers that showed significant variation between matched tumor and normal samples by 2DGE were identified by mass spectrometry. The validation steps involved a two-pronged approach: protein expression was evaluated at the mRNA level, and protein level expression was assessed using IHC and a high value TMA. Through a multi-step approach it is envisaged that one of the experimental anomalies will be circumvented. Using 2DGE, hnRNPK was identified as a potential biomarker which was further validated, through the use of semi-quantitative RT-PCR on a collection of colorectal sample pairs (normal *vs.* tumor). Moreover, the immunohistochemistry results have shown that hnRNPK has an aberrant subcellular localization in cancer cells being detected in both the cytoplasm and nucleus of CRC cells, whereas in normal colon the protein was exclusively nuclear, implicating hnRNPK as a diagnostic marker. Survival analysis showed that in Dukes' C colorectal cancer patients, stronger hnRNPK nuclear expression correlated with better prognosis, and hence holds the potential to be a prognostic indicator. The discovery of hnRNPK as a potential prognostic and diagnostic marker for CRC, verifies our chosen approach for biomarker research programs. Furthermore, this is enforced through another unique and novel set of biomarkers which have been published using the same discovery and validation approach (Coghlin *et al*., 2006).

Future Directions

It is clear from the findings discussed in this chapter that we have established a useful biomarker discovery platform. Our approach has been validated further by the uncovering of another potential biomarker using the same approach (Coghlin *et al*., 2006). Recent advancement in 2DGE tailored to simplify the complex proteome facilitates biomarker discovery, for instance, fractions enriched in membrane proteins or different compartments. Also, the discovery platform can be focused on other malignancies, for example, breast or lung cancer. With regard to findings in this study, it appears that cytoplasmic hnRNPK could potentially have a huge diagnostic value because it is solely detected in tumor tissues (primary tumors and lymph node metastasis). In one instance the diagnostic potential of hnRNPK could be utilized to diagnose colorectal cancers of ambiguous origin as it is frequent to encounter patients with metastatic adenocarcinoma for which the primary tumor site is unknown (Hillen *et al*., 2000). Knowledge of the primary site, where the tumor developed and spread from, facilitates appropriate clinical management and treatment of patients, as the disease prognosis and therapy vary. The diagnostic potential of hnRNPK could also be utilized for detecting early colonic tumor cases by the development of a population screening program to discriminate low and high risk individuals. Ideally, blood tests or stool samples from those who are examined could be used for population screening to intercept metastatic colon cancer cells. However, for the diagnostic potential of hnRNPK to develop further, it is necessary to demonstrate whether hnRNPK localization is exclusively anomalous in

colon cancer cells and not in other types of tumors. Importantly, hnRNPK levels also have to be examined in multiple centers representing different geographical areas and ethnic groups to reduce genetic and/or environmental contribution factors specific to this region. It is also worth noting that in this study hnRNPK showed a prognostic promise for late stage CRC which could be exploited by developing a prognostic test by immunohistochemistry staining in CRC solid tumors.

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4
Metastases and Recurrence of Colorectal Cancer: Diagnostic Role of Immunoscintigraphy

Vladimir Obradović and Vera Artiko

INTRODUCTION

Up to almost a half of all patients who undergo surgical resection of primary colorectal carcinoma can be expected to get recurrent or metastatic disease, predominatly within the first 2 postoperative years (Murphy *et al*., 1995). However, if the metastases and recurrences are identified before symptoms become evident, opportunities for a positive clinical outcome are enhanced. Barium enema examination and colonoscopy are two common modalities used in diagnosis of colorectal cancer, the latter being considered as a gold standard. Presently, advanced traditional and newly developed imaging techniques are also available. They are useful in staging the extent of such malignant tumors, including detection of their metastases and recurrences.

Anatomical imaging techniques, such as ultrasonography (US), computed tomography (CT), and magnetic resonance imaging (MRI), are the ones mostly used. Thus, for local staging, superficial tumors are best staged using endorectal US, which also provides an assessment of the tumor ingrowth into the rectal wall layers. More advanced local tumors are best imaged using MRI. Computed tomography is not very accurate in early detection and differentiation of a recurrence of colorectal carcinoma due to distorted local anatomy after surgery as well as in the case of radiation changes. On the other hand, clinical value of virtual colonoscopy, performed by new generation of CTs is still under investigation. As far as prediction of nodal status is concerned, none of the three mentioned imaging modalities can be reliably used in clinical decision making. This is because many affected lymph nodes are bellow 1 cm in diameter; thus, explaining poor sensitivity of these techiniques. Only MRI using a lymph node specific contrast seems promising in the detection of nodal disease. For the detection of distant metastases transabdominal US is often the first choice for liver examination, and development of contrast agents for this technique has significantly increased its potentials in detecting focal liver lesions. Chest X-ray is also used as one of the primary diagnostic tools. However, multidetector CT is the mainstay of staging and follow-up of these patients, because it provides good coverage of the liver, the complete abdomen and the chest in one session. MRI is commonly used as the definitive imaging modality in detecting and characterizing liver lesions.

On the other hand, functional imaging techniques, such as immunoscintigraphy (planar and SPECT modalities), as well as positron emission tomography (PET), provide a significant contribution to diagnosis of metastatic and recurrent disease, owing to their possible capacity to detect viable tumor tissue. Lastly, fusion techniques (PET/CT and SPECT/CT) have been introduced, providing information on both anatomical characteristics and viability of detected tumors. Although PET, mostly using ^{18}FDG, and particularly PET/CT, have great diagnostic potentials in all aspects of staging patients with metastatic and reccurent colorectal carcinomas, these techniques are not widely available yet, and such diagnostic methods are very expensive. This chapter deals with the usefulness of immunoscintigraphy in diagnostics of colorectal carcinomas.

GENERAL CONSIDERATIONS

Immunoscintigraphy (radioimmunodetection), as a variety of scintigraphy, belongs to nuclear medicine imaging procedures. It is mostly used for oncologic diagnostics, when it is based on specific radiopharmaceuticals, i.e., different monoclonal antibodies (Mabs) and their fragments against tumor-associated antigens, that are labeled with radionuclides (gamma emitters).

Following intravenous administration, such radiopharmaceuticals are accumulated in the tumor tissue, owing to their interaction with corresponding antigens. For successful imaging there is a need for the antigens to be expressed on tumor cell surfaces in > 100-fold concentration compared to normal tissues, and present in a quantity of at least 100,000 molecules per cell (Larson *et al*., 1984). Actually, target-to-background ratio should be at least 2:1, while ratios of 5:1 or more are necessary to detect deeper and smaller lesions.

Imaging is performed by registration of gamma radiation from the patient's body using a standard gamma scintilation camera (to obtain whole body and selective planar images) and/or single photon computerized tomography (SPECT) to obtain tomographic slices. Bearing this in mind, three major considerations in immunoscintigraphy are: the choice of the target marker, the choice of an appropriate radiopharmaceutical, and the choice of an effective imaging system.

Radiopharmaceuticals

Radiopharmaceuticals used for immunoscintigraphy are radiolabeled immunoreactive agents (intact Mabs and their fragments) against tumor-associated antigens.

Structure of Immunoreactive Agents

For the purposes of immunoscintigraphy, highly selective immunoreactive agents with particular specificity, charge, and stability are required. The specificity of an antibody (fragment) used refers to its ability to recognize a specific epitope in the presence of other epitopes of the same or different antigens, while the measure of the binding strenghth of an antibody (fragment) for a monovalent epitope is reffered as affinity. Presently, a large number of different Mabs are available in unlimited quanitities, each being produced artificially by a single B lymphocyte clone, using the hybridoma technique.

Mabs that are used for immunoscintigraphy mostly belong to IgG type

of immunoglobulins, each weighing ~ 150 kD. IgG is constructed of modules composed of two identical copies of both a heavy and light chain, which are held together by disulfide bonds, and the resulting molecule is often represented by a schematic Y-shaped molecule (Edelman, 1971). A total of ~ 1,200 amino acids are involved. Each antibody consists of two Fab fragments (fragments antigen binding) with specific immunoreactive areas that recognize the shape of epitopes on the surface of the antigen, and an Fc fragment that is more or less constant in structure and can be crystalized (fragment cristalline). The Fab fragment represents one third of the antibody molecule. Part of the Fab is relatively unchanged from antibody to antibody, while a second section, the "variable" region, varies among different antibodies. The variable regions of both chains bind together to form the antigen-binding domain. There are three hypervariable regions on the variable portion of the Fab, each being 5–10 amino acids in length, that constitute the actual epitope binding sites. In effect, the variable regions make up 25% of the amino acids of a Fab and, as such, allow an incredible number of interactive permutations.

Smaller immunoreactive agents (antibody fragments) are produced by enzymatic digestion. Thus, $F(ab')_2$ fragments are generated by the enzyme pepsin, resulting in the formation of a 100 kD bivalent protein molecule devoid of its Fc portion. The monovalent Fab fragments can be obtained by treating IgG with enzyme papain. The result are two 50 kD Fabs and one Fc polypeptide per IgG. Monovalent Fab' fragments can be obtained by treating $F(ab')_2$ fragments with mild reducing agents, such as cysteine. Even smaller immunoreactive agents are produced by protein engineering techniques, such as Fv (fragment variable) representing pairs of specific variable light- and heavy-chain regions, but as yet they have not been widely used.

Biodistribution of Immunoreactive Agents

Following i.v. administration, Mabs or their fragments are in constant "risk" of being removed from the vascular compartment before reaching the tumor tissue. To be more precise, normal tissues can also produce tumor-associated antigens. Moreover, in different tissues there are receptor-bearing cells or other sites capable of acquiring and destroying proteins. Generally, whole Mabs are normaly metabolized in the liver and reticuloendothelial system, whereas their fragments are principally cleared by the kidneys.

Intact antibodies are characterized by delayed clearance from the blood in relation to antibody fragments, maintaining lower target-to-background ratio, and exposing individuals to greater radiation if radiolabeled. Their approximate serum half-life is 48 h. Therefore, smaller immunoreactive molecules were proposed to be used in order to improve the clearance. In this way, the fragments $F(ab')_2$ have a serum half-life of between 24 and 36 h, and Fab' between 12 and 24 h (Halpern *et al.*, 1988).

Because tumor blood flow is much lower in relation to normal organ blood flow, the absolute tracer concentration within the lesion is reduced. Also, much of the blood may not even perfuse the tumor due to arteriovenous shunting. In addition, a small percentage of these agents can penetrate a tumor capillary. The larger the immunoreactive molecules, the shorter their

migration distance from the capillary wall. Also, a certain quantity of immunoreactive agents do not reach the antigens because of their possible variable expression. After repeated administration, because of their animal (mostly murine) origin, immunoreactive agents may interact with already produced specific human anti-murine antibodies (HAMA). The resulting immune complexes are removed from the blood by phagocytes of the reticuloendothelial system (RES), causing image degradation.

Radiolabeling of Immunoreative Agents

Mabs and their fragments are labeled with different radionuclides – gamma emitters, such as iodine-131 (^{131}I), iodine-123 (^{123}I), indium-111 (^{111}In) and technetium-99 m ($^{99\,m}$Tc). There are different techniques for radiolabeling of Mabs or their fragments, depending on the characteristics of radiolabels (Oriuchi and Yang, 2001). Thus, the process of labeling immunoreactive agents with ^{131}I or ^{123}I can be accomplished by lactoperoxidase technique, chloramine T reaction, Iodogen reaction, or Bolton-Hunter reaction. On the other hand, chelating agents, such as ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA), are most commonly used to chelate metallic cations such as ^{111}In and $^{99\,m}$Tc to intact Mabs or their fragments. The best results have been achieved with ^{111}In, with labeling efficiency of > 98%, while $^{99\,m}$Tc has shown much worse results for labeling of immunoreactive agents in this way. However, contrary to using any of chelation techniques, Fritzberg labeling method produces a $^{99\,m}$Tc-compound that is cleared rapidly from the vascular compartment and enters the tumor quickly, especially in the form of a Fab fragment. One should know that any *in vitro* radiolabeling method can cause alteration in the biological activity of Mabs, as it can destroy antibody integrity or interfere with antigen binding in the Fab region. Therefore, molecular damage at any stage of preparation can result in problems in biological effectiveness.

Improvements of Effectiveness of Immunoreative Agents

Given the fact that the uptake of immunoreactive agents in tumors is less than 0.1% of injected dose per gram of tissue (Goldenberg *et al*., 1990), causing low target-to-background ratio, there have been different approaches in improving immunoscintigraphic technique. Thus, administration of recombinant human interferons has shown an increase in the surface expression of specific tumor associated antigens recognized by Mabs. Also, a mixture (cocktail) of Mabs directed against different tumor associated antigens or against different epitopes of the same antigen can be successfully used to overcome the heterogeneity of antigen expression so as to improve tumor visualisation. By using a secondary antibody directed against primary labeled antibody, it is possible to get accelerated clearance of nontumour bound primary labeled antibody. On the other hand, alterations of the isoelectric point of the antibody molecule changes its distribution in the body. Using this approach, it might be possible to reduce the uptake of small fragments by kidneys. Locoregional administration of radiolabeled Mabs, through regional arteries or directly in pleural or peritoneal cavity, may improve tumor visualisation by increasing tumor uptake. Also, pretreatment with vasoactive immunoconjugates, that selectively alter vascular permeability

and/or blood volume of tumors *in vivo*, may significantly improve monoclonal antibody uptake in tumors. Special techniques for multistep *in vivo* labeling of Mabs and their fragments, based on high affinity and specificity of avidin–biotin and antibody–hapten interactions, were also developed and resulted in enhanced tumor imaging by drastically decreasing the background activity.

In order to reduce the HAMA reaction, there is a trend of using chimeric and humanized antibodies, instead of pure animal (murine) Mabs. They are produced by genetic engineering, and based on human Mabs with only specific immunoreactive parts of murine ones built into them. Intact human Mabs are also applied. However, they have not been widely used because of a very complicated procedure of their production.

Imaging Systems and Techniques

Immunoscintigraphic examinations are performed by a gamma scintilation camera, that provides possibilities for both spot views and whole body two-dimensional imaging. The advanced modality is SPECT system, providing three-dimensional presentation of the whole region, as well as evaluation of the cross-sectional slices.

Features and Functioning of Imaging Systems

A gamma scintillation camera with a dedicated computer system is the basic nuclear medicine imaging device. Following the administration of a radiopharmaceutical, a gamma camera externally registers the distribution of gamma ray emitting radionuclides in the body, using one or more detectors. The main part of the detector is a crystal (NaI), which scintillates in response to incident gamma radiation. When a gamma photon hits the crystal, an electron becomes loose from an iodine atom in the crystal. Then, as a result of its return to a minimal energy state, a flash of light is emitted. Light photons produced in the crystal strike the photocathode and eject loosely bound electrons into photomultiplier tube, where their acceleration and multiplication occurs. A complex electronic system collects, stores, and sums the counts, and according to spatial count density, displays the image on the monitor. The resulting two dimensional image reflects the distribution and relative accumulation of radioactive tracer elements in the organs and tissues in the field of view of a gamma cammera. In front of the crystal there is a collimator appropriate for different energies of radionuclides and types of examinations. It consists of an array of lead channels, perpendicular to the face of the crystal. It is capable of collimating radiation, allowing only the rays originating from the radioactivity directly in front and usually parallel to the tube axis to pass along the entire length.

Detectors are fixed to a gantry, and have the possibility to be moved around the bed on which the patient is positioned, thus allowing image acquisition of the same region in different positions: usually anterior, posterior, both lateral or (more rarely), anterior and posterior oblique at different angles. As a result, planar views of every region in this position can be obtained. Also, movement of the bed with constant speed allows detector(s) to monitor the distribution of radioactivity throughout the whole body.

The SPECT system is based on the gamma camera whose detectors rotate 360° around a particular region of the patient's body, acquiring images under

different angles. Afterwards, a computer reconstructs the collected data producing a three-dimensional image, as well as an evaluation of the cross-sectional slices. There are several methods used for data reconstruction in SPECT imaging, including preprocessing, back projection and post processing techniques. Also, different filters can be used.

Advantages of fusion imaging in combination with CT or MRI, and more recently, application of hybrid SPECT/CT systems are three-dimensional images of organs, monitoring a function and anatomy at the same time. These systems allow more precise estimation of the exact localization of the structures with higher uptake of radiopharmaceuticals, and distinguish them from the surrounding tissue.

Imaging Techniques

The aim of patient examination by immunoscintigraphy is to obtain the images of the "hot" spot(s), as a result of accumulation of radiopharmaceutical in the target tissue. Contemporary gamma cameras usually have both possibilities, performing whole-body acquisition and SPECT. Acquisition is performed with a large field of view detector, with a parallel holes high resolution collimator depending on the energy and count density of the photons of the radionuclides used. It is recommended that the device has more than one detector (usually two), in order to shorten investigations and diminish the possibility of patient movement. By shortening the procedure, it is also possible to perform multiple acquisitions of different regions, thus contributing to the entire investigation of the patient.

Firstly, at a particular time interval following the application of radiopharmaceutical, whole body acquisition is usually performed in order to detect possible foci of pathologic accumulation of the radiopharmaceutical in all regions of the body, which is especially important in detecting distant metastases. Then, selective planar images can be obtained. However, for detection/confirmation of small lesions, those not clearly visible on the planar imaging, SPECT of the particular region is suggested. SPECT offers improved contrast of the tumor in the section, differentiating it from the surrounding structures that may be overlapping in planar view.

IMMUNOSCINTIGRAPHIC METHODS USED FOR DIAGNOSTICS OF COLORECTAL CANCER

In order to achieve the best results of immunoscintigraphy application in diagnostics of different malignant tumors, including colorectal carcinomas, various investigations were performed. The aim was to obtain radiolabeled immunoreactive agents as well as imaging methods that provide rapid high-resolution imaging, high tumor-to-background ratios in all organs at risk of tumor recurrence or metastasis, and low immunogenicity and toxicity. A variety of radiopharmaceuticals used for immunoscintigraphy of colorectal tumors have their own characteristics regarding the time of the beginning of the acquisition, technical requirements regarding collimation, energy and window settings, as well as specific methabolic pathways predominantly due to the complex's stability; as well as employment of the whole antibody or its chosen fragments. Some authors have used immunoreactive agents developed in their own laboratories,

while others, including our group, have used available commercial kits.

Immunoscintigraphy Based on Radiopharmaceuticals Labeled with Radioiodines

Methods

In the early phase of immunoscintigraphic appllication for diagnostics of colorectal carcinomas, ^{131}I was the most commonly used radionuclide. It is characterized by half-life of 8 days, gamma emission of 360 keV, as well as beta-minus emission, and leads to high levels of patient exposure to radiation. In addition, its high energy gamma emission is not optimal for imaging using a gamma camera (the detector's crystal efficiency is ~ 20%), thus necessitating high-energy collimation. Before i.v., administration of the radioiodinated Mabs complex, nonradioactive solution of potassium iodide (Lugol's solution) must be administered in order to prevent significant thyroid uptake of radioiodine. Also, following administration, a quantity of radioiodinated Mabs can undergo dehalogenation in various normal tissues, especially in the liver. Additionally, it is not suitable for SPECT because of the heavy collimators used and low detection efficiency requiring prolonged time, necessitating application of small doses because of the heavy radiation exposure, the last of which further contributes to the duration of the study.

On the other hand, ^{131}I is easily available and inexpensive. Protein radioiodination is easy to perform and the complex of radioiodinated Mabs *in vivo* is stable. Saturation of physiologic iodine stores with nonradioactive iodine allows fast elimination of free radioiodine by the kidneys; thus, preventing significant thyroid or stomach uptake. Moreover, physical half-life of ^{131}I enables the study of the kinetics of uptake of different immunoreactive agents labeled with this radionuclide by target tissue over time. Acquisition should be performed 24 h to 7 days after administering ^{131}I labeled Mab fragments, and usually 24 h up to 12 days after administering ^{131}I labeled whole antibodies. Such radiopharmaceuticals are particularly useful for the detection of a neoplastic disease in the abdomen because of their low nonspecific uptake in the liver, spleen, and bone marrow.

One of such commercially available radiopharmaceuticals widely used earlier was *IMACIS 1*. It contained a cocktail of 111 MBq ^{131}I Mab 19-9 F $(ab')_2$ and Mab anti CEA $F(ab')_2$, and was infused over 30 min. Potassium iodide (600 mg/day) was administered orally for 10 days (starting 24 h before the injection) to block the uptake of free ^{131}I into the thyroid gland. Imaging was carried out after 96–120 h. Planar images (~ 6 min per image, or at least 200,000 counts over the whole field of view), including anterior and posterior projections of the thorax, abdomen, and pelvis, could be obtained using large field-of-view cameras, fitted with parallel hole high energy collimators. Because of the very low count rate, whole body scintigraphy, and particularly SPECT, were very difficult to perform. In order to achieve more precise estimation of the localisation of the pathologic lesions, as well as to increase target-to-background ratio, the dual isotope acquisition and subsequent subtraction of the obtained images are carried out. Actualy, images of vascular system (^{99m}Tc red blood cells/human serum albumin), the liver and spleen

(^{99m}Tc sulphur colloid) or the kidney (^{99m}Tc DTPA) are acquired and used for subtraction.

In relation to other isotopes of iodine, ^{123}I has more favourable characteristics, i.e., half-life of 13.3 h and 159 keV gamma emision, delivering small radiation dose to the patient. Application of ^{123}I for immunoscintigraphy allows the use of low-energy collimation. Unlike ^{131}I, it is not suitable for labeling with intact Mabs because they need acquisition to be delayed (even up to 36 h) in order to achieve high target-to-background ratio. After that period, longer time for planar acquistion is required, and particularly for SPECT, which is highly dependent on the count rate. On the contrary, when ^{123}I antibody fragments are used, such as Fab and Fab', earlier acquisition can be performed, as well as SPECT with very high count rate even with a small dose. Similarly to ^{131}I, there is a dehalogenation problem. One disadvantage of ^{123}I use is its high cost and limited availability. Mab fragments are usually labeled with an average of 130 MBq of ^{123}I and administered in a 60 min infusion. Pretreatment with potassium iodide (Lugol's solution) is necessary. Anterior and posterior images and a whole body scan should be performed at 1, 6, 24, and 48 h after administration. SPECT is recommended at 6 h in order to obtain transverse, coronal and sagital slices, using a filtered back-projection algorithm (60 views, 6°, 30 s/per image). When applied later, acquisition time should be prolonged for up to 24 h (60 s/view).

Results of Clinical Studies

Chatal *et al*. (1984), using ^{131}I labeled monoclonal antibodies 19-9, or its F(ab')$_2$ fragments, showed significant accumulation in 66% colorectal cancer sites. Baum *et al*. (1988) with ^{131}I labeled F (ab') $_2$ fragments of monoclonal antibodies against CA 19-9 and CEA ("radioimmunococktail" IMACIS 1) obtained high sensitivity (82%) and specificity (90%), especially in the diagnosis of pelvic recurrences and intra-abdominal metastases. Similarly, our group, using the same radiopharmaceutical, proved its value in the detection of recurrences in liver (Figure 4.1) and extrahepatic metastases (Obradović *et al*., 2006).

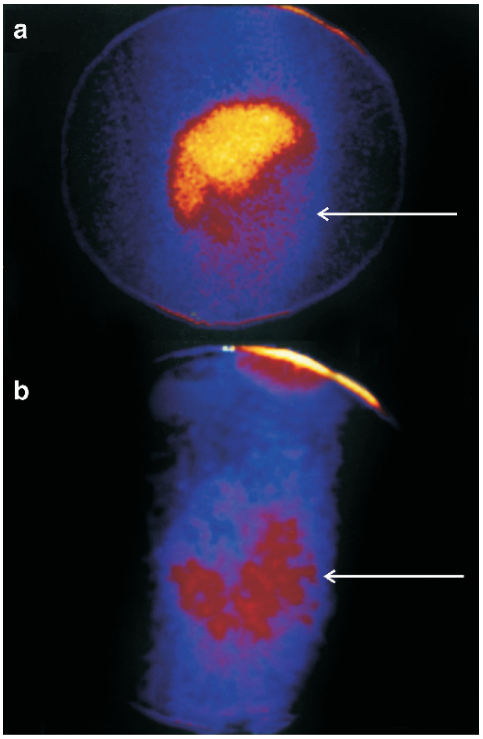

FIGURE 4.1. (a) Planar liver radiocolloid scintigraphy (abdomen, right lateral view): defect of accumulation in the anterior lower part of the right liver lobe (arrow). (b) Immunoscintigraphy with IMACIS 1 (abdomen, right lateral view): increased accumulation of activity in the anterior and lower part of the right lobe (arrow). Metastases of colon adenocarcinoma in the lower part of the right liver lobe
(Copyright permission, Hepatogastroenterology 2006;53:526–530, Obradović *et al*.)

Contrary to ours and the results of other authors, Holting *et al*. (1990) using immunococktail of ^{131}I labeled F(ab')$_2$ fragments of monoclonal antibodies against CEA, with Ca 19-9, found immunoscintigraphy results disappointing in comparison to other diagnostic modalities, especially concerning extrahepatic tumor diagnosis. Furthermore, accuracy could not be improved even by technical modifications such as changing of the antibodies, radiolabels or imaging techniques. Similarly, Schlag *et al*. (1987), using ^{131}I labeled CEA/Ca 19-9 antibodies, concluded that immunoscintigraphy cannot give information beyond that of conventional diagnostic tools for indication or planning of operative strategy in the treatment of recurrent colorectal cancer.

Goldenberg *et al*. (1993) found that immunoscintigraphy with ^{123}I labeled fragments, F(ab')2 and Fab', of IMMU-4, and anti-CEA monoclonal antibody (Immu-RAID-CEA) showed that this imaging method complemented CT findings by confirming suspected tumors and disclosing occult lesions with a very low possibility of developing HAMA. Also, Bischof-Delaloye *et al*. (1989) proved that immunoscintigraphy with SPECT based on ^{123}I-labeled anti-CEA Mab allows early detection of recurrence or metastasis of colorectal cancer, thus reducing the delay between diagnosis and treatment. Wong *et al*. (2004) evaluated an engineered intermediate-molecular-mass radiolabeled antibody construct directed against CEA (cT84.66). It demonstrated tumor targeting to colorectal cancer and a faster clearance in comparison with intact antibodies, making it appropriate for further evaluation as an imaging and therapeutic agent.

Immunoscintigraphy Based on Radiopharmaceuticals Labeled with ^{111}In

Methods

Indium-111 is also a pure gamma emitting isotope, with half-life of 67 h, and principal photons of 173 and 247 keV. However, although it has favorable physical characteristics for gamma camera imaging, it is not easily available and its use is expensive. For imaging with ^{111}In, medium-energy collimation should be used. Following administration, both ^{111}In labeled antibodies and this radionuclide in its free form can be partly accumulated in the liver, making it difficult to image tumor tissue in this organ. SPECT is also easily accomplished following the administration of ^{111}In-labeled antibodies. Transchelation occurs with consecutive high activity in the liver, spleen, and bone marrow. In the blood, ^{111}In is partly released from the antibodies and then bound to transferin. Urine and fecal excretions are very slow, and thus, high activity in blood pool and kidneys can also be observed.

The most widely used ^{111}In-labeled radiopharmaceutical for immunoscintigraphy is *OncoScint CR 103*. It is an immunoconjugate produced by site-specific modification of the monoclonal antibody B72.3. It is a murine immunoglobulin (IgG1) which is specific for glycoprotein (TAG-72) expressed by the majority of adenocarcinomas. The half life of ^{111}In approximates the biological half-life of B72.3. A radiopharmaceutical in a dose of 185–200 MBq is administered by slow injection for approximately 5 min. Anterior and posterior spot views of the abdomen, pelvis and/or chest (5×10^5 to 10^6 counts/view) can be obtained on two separate occasions at least 24 h apart, usually between 2 and 5 days

following the infusion. Also, posterior and anterior whole body imaging, using an acquisition speed of ~ 8–10 cm/min, can be performed. SPECT of abdominal and pelvic regions is nearly always performed. However, if the findings obtained by spot views or whole-body scintigraphy with regards to the extra-abdominal regions are suspicious for tumors, evaluation by SPECT is also required. Acquisition parameters include a 360° rotating orbit, sampling every 6° with an ~ 40 s acquisition per stop, using 128 × 128 (or 64 × 64) word matrix. A filtered back-projection algorithm is used for tomographic reconstruction of all three planes (transverse, coronal and sagital). Reconstruction is performed using Butterworth filter, order 6–10. Similarly to immunoscintigraphy with ^{131}I, dual isotope acquisition and subsequent subtraction of the obtained images are carried out. Thus, images of vascular system (^{99m}Tc red blood cells/human serum albumin), the liver and spleen (^{99m}Tc sulphur colloid) or the kidney (^{99m}Tc DTPA) are also acquired and used for subtraction.

Another widely distributed and commercially available radiopharmaceutical, *INDIMACIS 19.9*, contains 19.9 F(ab')$_2$/DTPA monoclonal antibody fragments. A dose of 185 MBq of this radiopharmaceutical should be slowly infused intravenously with 100 ml of 0.9% injectable solution of sodium chloride over 30 min. Equipment, technical details, acquisition time regarding the onset, duration and the modality of acquisition and reconstruction are the same as above.

Results of Clinical Studies

One of the most widely used commercially available radiopharmaceuticals for immunoscintigraphy is *OncoScint CR 103*. Concerning the results of OncoScint the sensitivity of the method depends on the density of TAG-72 antigen expression of the particular tumour, but no current *in vivo* method is available for its estimation. Volpe *et al*. (1998) claim that the combination of ^{111}In-CYT-103 and CYT-37 improved the sensitivity of immunoscintigraphy for the detection of colorectal cancer compared to that obtained with a single Mab imaging. This cocktail-antibody imaging may enhance staging and management of patients with colorectal carcinoma. Both false-positive and false-negative studies are seen in > 10% of patients (Tempero, 1993). Initial staging of primary colorectal carcinoma has been studied with preoperative use of this agent in addition to standard procedure (Winzelberg *et al*., 1992). Apart from high sensitivity of both planar immunoscintigraphy (16/23), and SPECT (21/23) in the diagnosis of primary lesions, and SPECT in the detection of lymph node metastases (3/5), false-positive scans were also reported. Nabi *et al*. (1995) strongly recommended SPECT in all patients undergoing immunoscinti-graphy, because it identified tumors missed on planar scans in 35% of patients, and provided additional information regarding tumor burden in 23% of patients. Neal *et al*. (1996) indicated that there is a significant difference in uptake ratios between patients with carcinomatosis and those without it, and that quantitative analysis can be a useful adjunct to visual interpretation. According to Dominguez *et al*. (1996), immunoscintigraphy with ^{111}In-CYT- 103 was more accurate compared with a CT scan, but when the value was examined with respect to its potential contribution to patient management, it was beneficial in only 13% of patients. Some authors

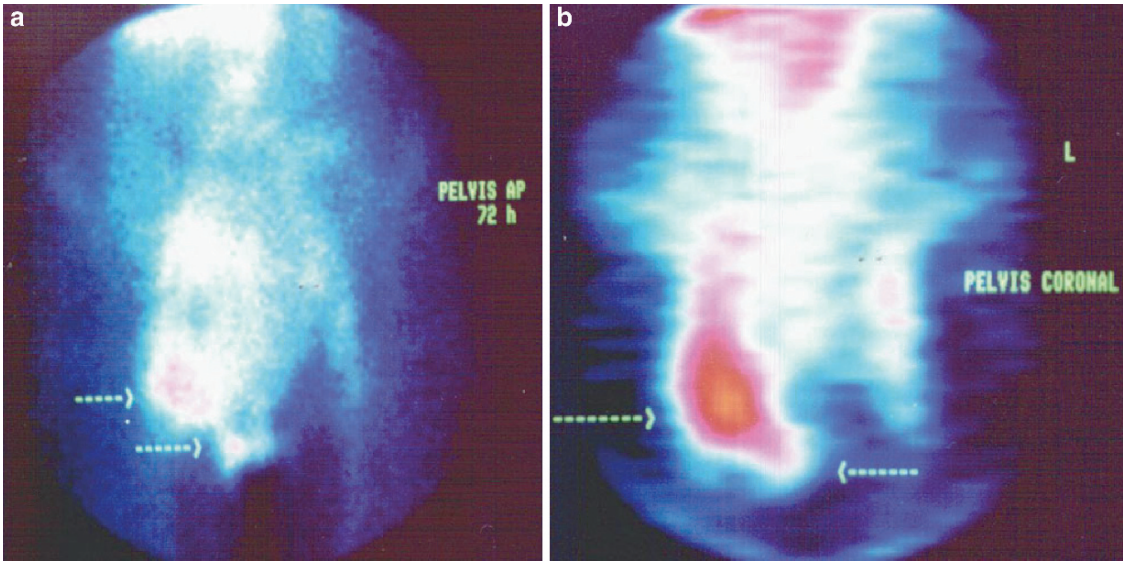

FIGURE 4.2. (a) Planar immunoscintigraphy (OncoScint), (pelvis, anterior view): area of increased accumulation of the radiopharmaceutical in the right iliac fossa (arrows). (b) SPECT immunoscintigraphy (OncoScint), (pelvis, coronal): much more visible area of increased accumulation of the radiopharmaceutical in the right iliac fossa (arrows). Recurrence of colon adenocarcinoma

show cases of recurrence of colorectal carcinoma not detected by MRI and CT. Goldenberg (1997) point out a particular application of these antibodies in disease staging and disclosure of occult lesions. The results of Doerr *et al*. (1991) recommended the procedure for presurgical evaluation of colorectal cancer patients. According to our previous results (Artiko *et al*., 2003), OncoScint scintigraphy is a sensitive method for the detection of local recurrence (Figure 4.2) and extrahepatic metastases (Figure 4.3) in colorectal carcinoma and has an important role in therapeutic decision making process. In adition, this radiopharmaceutical proved its clinical value in the detection of liver metastases and viability assessment after radiotherapy and surgery (Obradović *et al*., 2006). SPECT improved the sensitivity of the method, although small recurrences can sometimes be overlooked. Because of the cost of the procedure, Ryan (1993) has

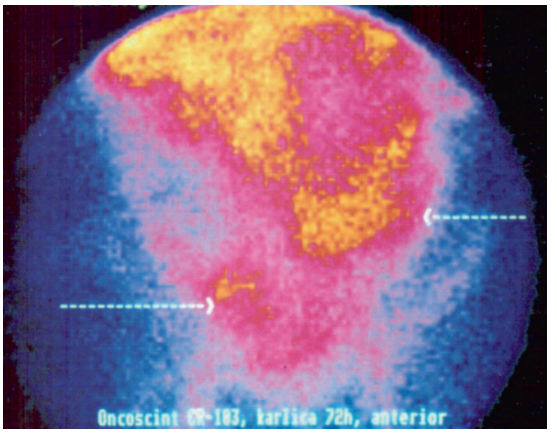

FIGURE 4.3. Planar immunoscintigraphy (OncoScint), (abdomen, anterior view): diffusely increased accumulation of the radiopharmaceutical throughout the abdomen (arrows). Peritoneal metastases of colorectal carcinoma
(Copyright permission, Hepatogastroenterology 2003;50:1029–1031, Artiko *et al*.)

suggested that immunoscintigraphy with this agent should be performed in patients with particularly aggressive but apparently localised tumours or patients with

indeterminate findings on standard initial staging in whom the surgical or adjuvant chemotherapeutic approach might be altered if metastasis has been known to be present.

Some authors performed immunoscintigraphy with radiolabeled monoclonal antibody fragments. One of them is *INDIMACIS 19.9*, containing 19.9 F (ab')$_2$/DTPA monoclonal antibody fragments. Our results with this radiopharmaceutical (Artiko *et al*., 2003; Obradović *et al*., 2006), are very similar to the results obtained with OncoScint; i.e., it proved its clinical value in the detection of recurrences, metastases (Figure 4.4), and viability assessment after therapy, especially using SPECT. Chetanneau *et al*. (1990) confirmed the advantage of immunoscintigraphy using ^{111}In labeled carcinoembryonic antigen (CEA)-specific and/or 19-9 F(ab')$_2$ fragments over conventional methods, and especially so in the diagnosis of pelvic recurrences. In order to improve sensitivity of the method, keeping in mind that a major drawback of ^{111}In-labeled monoclonal antibodies (Mab) was the presence of intense liver, renal, and bone marrow nonspecific activity, Liehn *et al*. (1989) introduced the subtraction imaging method. It included a simple algorithm for determining the limits of the color scale based on count density in the iliac crest.

Some experiences with ^{111}In labeled intact murine monoclonal antibodies in colorectal cancer suggested that immunoscintigraphy images hepatic metastases poorly (Moffat *et al*., 1999), and an antimurine immune response was frequently provoked. Apart from developing antibodies to murine immunoglobulin (33% of patients) administration of OncoScint can induce adverse effects (Doerr *et al*., 1991), primarily fevers and itching.

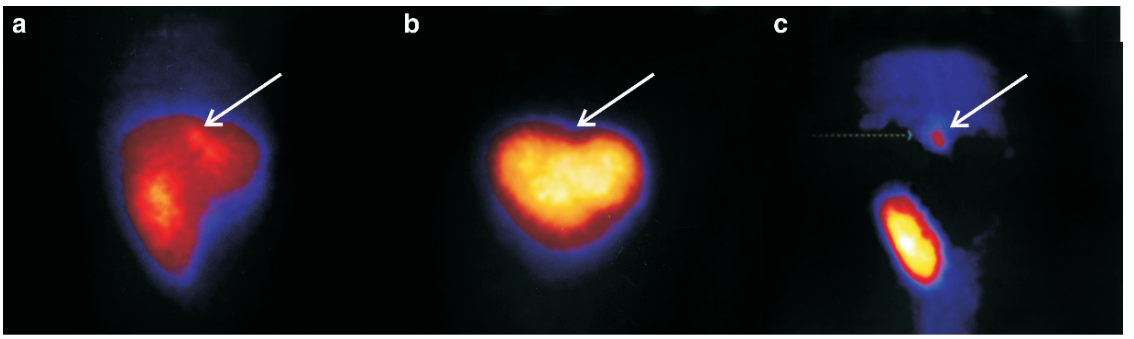

FIGURE 4.4. (a) Immunoscintigraphy (INDIMACIS 19.9), (abdomen, right lateral view): "hot spot" diameter 20 mm point out increased accumulation of radioactivity in the upper part of the right liver lobe, on the upper edge (arrow). Non-specific accumulation of the radiopharmaceutical in the right kidney. (b) Planar liver radiocolloid scintigraphy (abdomen, right lateral view): defect of accumulation ("indent") in the upper border of the right liver lobe (arrow). (c) Subtraction image (a–b): Solitary "hot" spot (20 mm) in the upper part of the right liver lobe (arrow). Non-specific activity in the right kidney. Metastasis of colon adenocarcinoma in the upper part of the right liver lobe
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Immunoscintigraphy Based on Radiopharmaceuticals Labeled with ^{99m}Tc

Methods

The most commonly used radionuclide in nuclear medicine is ^{99m}Tc, with half-life of 6 h and gamma photons of 140 keV, thus delivering very small radiation dose to the patient. The advantages of ^{99m}Tc-labeled radiopharmaceuticals are the lowest price, simple procedure for labeling and its ready availability as a generator-produced on-site. Imaging with ^{99m}Tc is possible with low-energy collimation. Because of the short-half-life, acquisition must be performed within the first 24 h. Thus, it is limited to the use of Fab or Fab' fragments because $F(ab')_2$ and intact Mab theoretically are not cleared fast enough from the vascular compartment to reach satisfying target-to-background ratio and allow acquisition of diagnostic images. High renal and gastrointestinal acumulation of ^{99m}Tc-Fab makes abdominal imaging difficult. However, its physical properties allow application of higher doses of radiopharmaceuticals, enabling faster imaging, better target-to-background ratio and good imaging statistics for SPECT. Images are usually performed after 10 min (blood pool visualisation), 2–5 h and/or 18–24 h after application. When labeled monoclonal antibody fragments are used, it is more important to acquire early (2–5 h) images, while the delay can be avoided. If intact Mabs are used, early images can be skipped, but it is mandatory to perform delayed ones. Bowel activity can obscure late images, so they should be interpreted with caution.

The most widely used ^{99m}Tc labeled radiopharmaceutical for this purpose is *CEA-Scan,* that comprises an antibody fragment (Fab') against carcinoembryonic antigen (CEA, IMMU-4). A dose of 740 – 1, 110 MBq of ^{99m}Tc labeled *CEA-Scan* is administered and images are obtained 2–5 h following the administration. Imaging is performed with a large field of view gamma camera equipped with a parallel hole low energy collimator. Posterior and anterior whole body imaging using an acquisition speed of 8–10 cm/min should be performed. Alternatively, it is possible to obtain anterior and posterior spot views of the abdomen, pelvis and chest. Image acquisition should be set at 10 min per view (5×10^5–10^6 counts). If needed, delayed images of the extra-hepatic abdomen should be acquired for a preset time of 15 min at 18–24 h post-injection. SPECT of the pelvis and abdomen is performed using a 360° circle, with a maximum of 6° steps, acquiring images for 30–40 s per stop The data are processed using a filtered back-projection algorithm, and all three planes (transverse, coronal, and sagital) are reconstructed. Reconstruction is performed using Butterworth or low pass filter with the order set between 6–10. Delayed 24 h images, spot views, are indicated only when there is equivocal abnormal uptake seen on early planar images in the extra-hepatic abdomen that could be bowel activity. Actually, a normal bowel activity will move, disappear, or change shape on the delayed scan, while abnormal uptake will remain fixed on both early and late images.

Acquisition and processing using ^{99m}Tc labeled Mabs is done in a similar way, although some authors suggest imaging 10 min after application of radiopharmaceutical as well as the delayed images (18–24 h). One of the more widely used ^{99m}Tc radiopharmaceuticals is *Scintimun CEA* (anti CEA Mab 99 mTc-BW 431/26).

There are other ^{99m}Tc radiopharmacuticals that are used (though not as widely) with almost the same acquisition procedure. Thus, Oliva *et al*. (2001) developed IOR C-5, a G1 immunoglobulin type intact murine Mab, which demonstrated a significant affinity for the epithelial tissues, leading to its use in a pilot clinical study to perform a immunoscintigraphy of the colorectal primary tumors and their locoregional recurrences. The other antibody (Ior-CEA1 Mab), used by the same authors (Oliva *et al*., 2005), is directed against a specific carbohydrate epitope on cell bound and free CEA. Application of higher doses of ^{99m}Tc allows SPECT images after 18–24 h (30 s per view, 360°).

Results of Clinical Studies

^{99m}Tc labeled radiopharmaceuticals for immunoscintigraphy can be labeled either antibody fragments (*CEA-Scan*, etc.) or whole antibodies (*Scintimun CEA*, etc.). According to Oriuchi (1999), the results using ^{99m}Tc labeled anti-CEA monoclonal antibody have shown particularly promising as means of whole body imaging in patients with colorectal cancer. Moffat *et al*. (1999), found that the sensitivity of this method was superior to that of conventional diagnostic methods (CT) in the extrahepatic abdomen and pelvis, while it complemented the conventional ones in the liver. The positive predictive value (98%) and imaging accuracy is particularly present in occult cancer (61%) when both imaging methods were included. They concluded that this method affords high-quality, same-day imaging, uses an inexpensive and readily available radionuclide, and adds clinically significant information in assessing the extent and location of the disease in colorectal cancer patients. Similarly, García Vicente *et al*. (2002) achieved the values of sensitivity, specificity, positive and negative predictive value for the immunoscintigraphy with *CEA-Scan* of 91%, 76%, 77%, and 90%, respectively, higher than using CT and CEA blood level. According to Behr *et al*. (1997) lesion-based sensitivity of immunoscintigraphy with this radiopharmaceutical was 94%, diagnostic accuracy 92%, both being unrelated to the CEA-serum level. The results indicate that immunoscintigraphy together with SPECT can achieve reliable and sensitive localization of tumor lesions. The combination of immunoscintigraphy with conventional imaging techniques can noninvasively improve the estimate of surgical resectability. Similarly, Moffat *et al*. (1999), using CT plus immunoscintigraphy in patients with recurrent or metastatic colorectal cancer improved the correct prediction of resectability by 40% as well as of unresectability by 100%, compared with CT alone. They added that immunoscintigraphy should be used in combination with conventional modalities to contribute to diagnostic accuracy in patients with known or suspected recurrent disease. *CEA-Scan* rarely induces a HAMA response (Moffat *et al*., 1999).

Some authors presented their results with *Scintimun CEA*, and concluded that immunoscintigraphy is useful in patients with colorectal carcinoma, especially in case of recurrences and it is a complementary technique to other diagnostic procedures. Poshyachinda *et al*. (1996) obtained the 87% overall accuracy of immunoscintigraphy using *Scintimun CEA* in the diagnosis of recurrent colorectal carcinoma. Its sensitivity in the detection of locoregional or abdominal recurrence and liver metastases was 97% and 89%,

respectively. This imaging method was more accurate than a CT scan in the detection of pelvic recurrence and liver metastases, while a CT scan was superior in detecting lung metastases. They concluded that immunoscintigraphy is most useful in patents with rising CEA levels on clinical follow-up while the other diagnostic investigations are negative. The advantages of immunoscintigraphy include the ability to detect tumor recurrence prior to other investigations and to identify tumor recurrence in areas such as the pelvis, where CT and MRI have their greatest weaknesses. The imaging accuracy is significantly increased when combined CT and antibody imaging is performed.

Apart from some of the above mentioned, usually commercially available, radiopharmaceuticals, certain authors used either whole monoclonal antibodies or antibody fragments that are not widely available. According to Granowska *et al*. (1989) monoclonal anti-CEA antibody PR1A3 reacts strongly to both well and poorly differentiated colorectal carcinomas and has advantages over other colorectal epithelium-reactive antibodies because its antigen appears fixed to the tumor and does not appear in the lymphatics or normal lymph nodes draining a tumor. PR1A3 reacted with 59/60 colorectal tumors (Richman and Bodmer, 1987), whereas CEA reactive B72.3 with only 75% (Salvatore *et al*., 1989). With ^{99m}Tc labeled PR1A3, no adverse effects or thyroid uptake was observed. All primary colorectal cancers were all image positive. In the assessment of recurrent tumor in the abdomen or pelvis, the accuracy was (94%), including true-positive findings in some cases whose serum carcinoembryonic antigen was normal. There was a positive predictive value for abdominal or pelvic recurrence of 92% and a negative predictive value of 100%. In those patients whose liver was able to be evaluated, the accuracy was 91%. There was a positive predictive value for liver metastases of 88% and a negative predictive value of 93%. High uptake was seen in the undifferentiated cancer.

Lunniss *et al*. (1999) with ^{99m}Tc-radiolabelled PR1A3 scanning obtained sensitivity for recurrent colorectal cancer 96%, specificity 50%, positive predictive value 73%, and negative predictive value 89%. In 16/40 patients, the interpretation of the findings either strengthened the management decision or altered the management.

Oliva *et al*. (2001) with IOR C-5 obtained good results in patients who were suffering from colorectal cancer or in those with a suspicion of recurrence. The same authors (Oliva *et al*., 2005), using Ior-CEA1 Mab, detected primary colorectal malignant tumors, their recurrences and metastases with high sensitivity. SPECT improved the diagnosis in patients with occult liver metastases or suspected pelvic recurrences. They concluded that immunoscintigraphy findings can help clinicians to modify the treatment plan and select optimal therapy. With the same antibody, Sirisriro *et al*. (2000) proved 86% sensitivity, 71% specificity, 83% accuracy, 94% positive predictive value, and 50% negative predictive value for the detection of colorectal cancer. Fifty-two percent of the immunoscintigraphic findings provided more information than computed tomography with clinical impact on further management. He concluded that ^{99m}Tc-IOR-CEA1 scintigraphy is a promising investigative method which is safe and has a high degree of accuracy in the detection

of recurrent colorectal carcinoma, especially in those patients whose serum-CEA and computed tomography findings are equivocal for recurrent diseases.

Comparison of Diagnostic Values of Different Radiopharmaceuticals

In order to achieve the best results and bearing in mind drawbacks of different radiopharmaceuticals, some authors tried to use several radiopharmaceuticals in the same study and compared the results. Thus, Bares *et al*. (1989) who used antibody preparations (^{99m}Tc labeled complete anti-CEA antibodies – BW 431/26, ^{111}In labeled F(ab')$_2$-fragments against CEA-BW 431/31, and a mixture of ^{131}I labeled F(ab')$_2$-fragments against CEA and CA 19-9-IMACIS-1), yielded equal diagnostic sensitivities (65%, range 60–78%), except for liver metastases. Best results were gained in local recurrences of gastrointestinal cancer (12/15 true positives), most of them exclusively recognized by immunoscintigraphy. Similarly, Leitha *et al*. (1990), with three different anti-CEA Mab (^{111}In /^{131}I BW 431, ^{131}I IMACIS-1, ^{99m} Tc BW 431/26) obtained global sensitivity ranging from 64–73%. All Mab underestimated the extent of liver involvement, and showed a high sensitivity in imaging local recurrences ranging between 50% for the ^{131}I IMACIS-1 and 100% for the ^{99m} Tc BW 431/26. In addition, Riva *et al*. (1989), with monoclonal antibodies anti-CEA F(ab') $_2$, labeled with ^{131}I or ^{111}In detected all primary tumors and almost all of their associated lesions, most of them previously undetected, allowing an improvement in patient staging before surgery. The best outcomes were obtained for abdominal and pelvic recurrences and lymph node lesions, while the lowest levels of sensitivity were observed for liver metastases.

The results by Herry *et al*. (1987), who evaluated a mixture of anti-CEA and anti-19.9 F(ab') $_2$ fragments in the investigation of colorectal carcinoma, showed that the pelvic recurrence was the best indication for this investigation. Similarly, Buraggi *et al*. (1987) used monoclonal antibody to CEA (F023C5), obtained by cell fusion technique. F(ab')$_2$ fragments were subsequently prepared and labeled with ^{131}I and ^{111}In. The best results were obtained in the detection of the tumors of the gastrointestinal tract and the worst in the detection of liver metastases. Muxi Pradas *et al*. (1996) performed immunoscintigraphy with anti-CEA Mab ^{99m}Tc-BW 431/26 (group I) and antiTAT-72 Mab ^{111}In-CYT-103 (group II). The sensitivity in the diagnosis of primary tumors in group I was worse than in group II (54.2% vs. 66.7%). If rectum tumors were excluded, the sensitivity increased to 80% and 85.7%, respectively. In the suspicion of recurrences, if only lesions confirmed at surgery were considered, the sensitivity was 75% in group I and 89.7% in group II. Immunoscintigraphy has been the only technique able to diagnose recurrences in 4/23 cases from group I and 14/32 from group II. However, the results regarding liver metastases were not so encouraging. No relationship was found between tumor markers levels and the immunoscintigraphic result. They concluded that immunoscintigraphy is useful in patients with colorectal carcinoma, especially in cases of recurrences and it is a complementary technique to other diagnostic procedures. Ychou *et al*. (1989) proved, using anti-ACE antibodies labeled with ^{131}I, ^{123}I

or ^{111}In, that immunoscintigraphy is less sensitive than both US and CT for localizing hepatic metastases, but also that the best indication for this method remains the diagnosis of pelvic recurrences.

Immunoscintigraphy and Positron Emission Tomography

Positron emission tomography (PET) uses short living positron emitting radionuclides. The technique is based on registration of two gamma photons emitted simultaneously 180° from one another, originated by annihilation of the positrons. The simultaneous use of a large number of detectors positioned in a ring around the patient (PET scanner) enables coincidence detection of the arrival of the two photons and location of their origin. The most widely used PET radiopharmaceutical in oncology is ^{18}F labeled fluorodeoxyglucose (FDG).

Some investigators tried to compare the results obtained by PET and immunoscintigraphy. According to Ito *et al*. (1997) the usefulness of PET and immunoscintigraphy (by means of ^{131}I or ^{111}In anti-CEA monoclonal antibody) was confirmed for the diagnosis of recurrent colorectal cancer. They concluded that, although PET reflects the biological character of tumor and makes a more accurate diagnosis by combined use with regular CT and MRI, this technique cannot provide the specificity of an antibody based functional imaging agent, and cannot help in selecting patients for the antibody-based therapy. However, Willkomm *et al*. (2000) point out that both FDG PET and ^{99m}Tc-labeled anti-CEA Fab' are suitable for the diagnosis of local recurrence of colorectal carcinoma, but that FDG PET is clearly superior in the detection of distant metastases (liver, bone, lung) and lymph node involvement. There are also attempts of using PET radiopharmaceuticals for immunoscintigraphy of colorectal carcinomas, mainly using ^{68}Ga labeled antibodies.

Radioimmunoguided Surgery

Recently, immunoscintigraphy is being combined with gamma detecting probe-guided surgery of colorectal carcinoma. It is based on the concept of sentinel-node-diagnosis, and is just being clinically evaluated. Lechner *et al*. (2000), applied ^{99m}Tc – CEA Scan to patients 24 h before surgery. During surgery, the radioactivity in the lymph glands surrounding tumors was measured with a gamma detecting probe and compared to much lower-activity in healthy nodes. All lymph nodes of interest were then excised and submitted to frozen section pathology. Thus, in 30% of cases this method led to an up-staging of the disease. Furthermore, metastatic spread to lymph nodes was not regionary for the primary tumor. According to them, this is the way to precisely identify even very small tumor deposits, leading to accurate staging even during surgery. Radioimmunoguided surgery is found to be particularly useful in recurrences and in small tumour deposits which are difficult to localise. Hladik *et al*. (2001) concluded that both immunoscintigraphy and radioimmunoguided surgery enable one to make a more accurate diagnosis. While treating the primary disease, the use of radioimmunoguided surgery may help in assessing the necessary extent of operation performance, as well as in staging of the disease by revealing occult lymph nodes involved. Preoperative immunoscintigraphy seems to be a useful diagnostic method for the detection of

tumor recurrence. According to Florio *et al*. (2002) radioimmunoguided surgery was performed in all, while immunoscintigraphy in 95% of cases. One case, which was negative at immunoscintigraphy, was found to be positive intraoperatively. According to the authors, radioimmunoguided surgery is a useful technique, but needs to be validated in larger samples.

CONCLUSION

Despite a variety of investigations related to the application of immunoscintigraphy in diagnostics of colorectal cancer, using different radiolabeled immunoreactive agents, there has not been a consensus among the investigators regarding the best modality(ies) of the method, including the specific radiopharmaceutical(s) for this purpose. Each modality has its characteristic advantages and disadvantages. However, some general conclusions concerning potentials of immunoscintigraphy in such diagnostics can be made.

The main advantages of immunoscintigraphy are possibilities of estimating tumor tissue viability, as well as of the whole body and tomographic (SPECT) imaging at the same time. Therefore, immunoscintigraphy is a very suitable method for staging the extent of colorectal cancer before and follow up of such patients after surgery, radio- and chemotherapy, being mainly indicated in cases suspected of tumor metastases or recurrence.

Suspicion of pelvic recurrence of such tumor is considered by many investigators as the most important indication for application of immunoscintigraphy, because other, especialy anatomical imaging methods, are significantly limited in their ability to explore this region. Concerning colorectal cancer, immunoscintigraphy contributes very much to the detection of extrahepatic abdominal metastases, and is complementary with anatomical imaging methods in detection of liver metastases. Its application is also indicated in cases of inconclusive outcome of routine diagnostic workup, as well as in patients in whom the barium enema examination and colonoscopy cannot be performed. As a complementary method, it can be useful in assessing the resectablility of the tumor. In adition, radioimmunoguided surgery has been advocated as a method of more accurate detection of tumor extension and accomplishing radical resection.

In general, disadvantages of immunoscintigraphy can be low resolution, small target-to-background ratio, and nonspecific uptake of the radiopharmaceutical in different organs and tissues. However, with SPECT one achieves better distinction of tumor in comparison to other structures and estimation of its size, all of which allow for a more accurate diagnosis and assessment of localisation, as well as discovery of smaller lesions. Furthermore, the hybrid SPECT/CT systems, that have recently been introduced, are expected to significantly improve the potentials of the both included modalities in diagnostics of malignant tumors, owing to the possibility of combined anatomical/functional imaging to produce fusion images. Considering immunologic response to the agents used, they may be one of the major drawbacks of the method. However, it is important to emphasize that although HAMA reaction is very rare, it can to a certain extent limit performance of repeated studies, as well as of the corresponding radioimmunotherapy.

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5
Colorectal Cancer Diagnosis Using DNA Levels in Blood and Stool

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the fourth most common cause of death from cancer worldwide. Each year, there are more than 1 million new cases diagnosed and more than 500,000 deaths from this disease (Parkin *et al*., 2005). Generally, the timescale for the development of a premalignant lesion into cancer is 5–10 years (Davies *et al*., 2005), which means that there is a large potential to reduce the burden of the disease by diagnosing lesions in precancerous or early cancer stages. Moreover, considering its characteristics (long cancer development interval, location within an accessible organ and high lifetime incidence), CRC is suitable for mass screening programs, and it has been estimated that > 50% of deaths due to this disease could be prevented by screening tests (Walsh and Terdiman, 2003). Apart from the fecal occult blood test (FOBT), the majority of currently available screening tools are invasive and expensive, and there is an ongoing debate regarding the best and most cost-effective method to use in screening programs for CRC.

A potentially important diagnostic tool is the analysis of molecular alterations detectable in human DNA extracted from stool, and many authors have investigated this area by analyzing a single molecular target or a combination of these (Ahlquist *et al*., 2000; Rengucci *et al*., 2001; Imperiale *et al*., 2004; Itzkowitz *et al*., 2007). Unfortunately, the vast majority of these methods are time-consuming and expensive, and it is highly unlikely that they will be transferred to clinical practice for early diagnosis programs.

The molecular approaches that are relatively inexpensive and easy to perform are the analysis of genomic DNA in biological fluids, including the evaluation of free genomic DNA, present in plasma or serum (Flamini *et al*., 2006; Umetani *et al*., 2006a), and the quantification of genomic DNA fragments in stool (Calistri *et al*., 2004; Zou *et al*., 2006). In this chapter we briefly review current diagnostic approaches and describe in detail the molecular approaches based on genomic DNA evaluation in biological fluids.

CURRENTLY AVAILABLE TESTS AND THEIR COST-BENEFITS

Fecal Occult Blood Test

Colorectal polyps or cancers have surface blood vessels that are often easily damaged by the passage of feces, leading to the release of a small amount of blood. The FOBT was developed to detect this blood using different methodologies. The most commonly used approach is based on the measurement of hemoglobin peroxidase activity. However, the guaiac-based fecal occult blood test has low specificity and relatively low sensitivity for identifying colorectal neoplasia (Morikawa *et al*., 2005). In fact, other diseases such as hemorrhoids, anal fissures, colon polyps, peptic ulcers, ulcerative colitis, gastroesophageal reflux disease, and Crohn's disease can also result in the presence of blood in stool, thus determining a positive FOBT result. Moreover, some foods or drugs can affect the result of the test, e.g., aspirin or nonsteroidal anti-inflammatory drugs (NSAIDs), vitamin C excessive intake, red meats, and some vegetables and fruits (raw broccoli, cauliflower, horseradish, parsnips, radishes, turnips and melons).

A new alternative test, the immunochemical fecal occult blood test (iFOBT), also detects occult blood in stool. This test is based on the detection of human hemoglobin molecules, with a consequently more specific identification of colorectal lesions, and does not have dietary confounds (Levi *et al*., 2007), which probably makes it easier to use. Moreover, it is not only possible to detect the presence of occult bleeding but also to quantify the amount of blood present in feces. Obviously, even this test may not detect a tumor that is not bleeding, and it is therefore preferable to perform multiple stool sampling and testing to have a more accurate result (Levi *et al*., 2007). Furthermore, other diseases that result in the presence of blood in feces may also cause false-positive results. The most important advantages of iFOBT are its non-invasiveness and high compliance, which permits its use in mass screening programs. The disadvantages are its low sensitivity and, in the event of a positive result, the need for a colonoscopy to further investigate and verify such positivity.

Flexible Sigmoidoscopy

A sigmoidoscope is a slender, flexible, lighted tube, which is inserted through the rectum and into the lower part of the colon. Unfortunately, it is only possible to reach the junction of the descending colon and the sigmoid colon or, at most, the splenic flexure. Although any small polyps found can be removed, the detection of an adenomatous polyp or CRC makes it necessary to perform a colonoscopy to look for further polyps or cancerous lesions in the rest of the colon. Furthermore, as this method is an instrumental analysis, patients must first undergo a bowel preparation to clean out the lower colon, which can cause discomfort. Sigmoidoscopy is capable of detecting distal colon tumors with very high sensitivity and specificity (97% and 94%, respectively) (Winawer, 2003). Some case-control studies indicate a 50–95% reduction in mortality from distal (left-sided) CRC in individuals who undergo a single sigmoidoscopic screening (Newcomb *et al*., 2003).

Colonoscopy

A colonoscope is capable of examining the entire colon, permitting a more accurate analysis compared to sigmoidoscopy. The technique is reported to be able to detect CRC with > 95% sensitivity and close to 100% specificity (Winawer, 2003), and also has good sensitivity for identifying adenomas. If a large polyp or other abnormality is detected, a biopsy is taken and in some cases a complete eradication of the disease may be achieved during the test. Although colonoscopy is generally a safe procedure, the scope can, on occasion, perforate the bowel, causing serious complications that sometimes require surgical repair. The need for bowel preparation and highly skilled operators represent the main obstacles to the adoption of the technique as a first-line screening modality (Davies *et al*., 2005).

Barium Enema with Air Contrast

This procedure, also called a double contrast barium enema, is a radiological technique that enables visualization of the entire colon after rectal administration of barium followed by air, and permits the identification of large polyps and cancers. If an abnormality is seen, colonoscopy is recommended. An important technical limitation of this method is its poor sensitivity, especially for polyps.

Virtual Colonoscopy

This test is more accurate than the barium enema but not as good as colonoscopy for finding very small polyps. Its advantages lie in the fact that it has a rapid execution time and does not require patient sedation, while its main drawback is that any polyp or growth found must be biopsied by other endoscopic methods. High patient acceptability and the relative safety of virtual colonoscopy might ultimately result in it being preferable to endoscopy, especially if costs can be kept low, for example, through software advances that reduce the time needed for image interpretation. Moreover, this method is indicated for patients who cannot undergo colonoscopy, such as those with obstructing tumors or those who are at high-risk of complications from colonoscopy (Davies *et al*., 2005).

IN SUMMARY

It is concluded that endoscopic screening examinations have an exceptionally high potential to reduce CRC incidence and mortality by early detection of colorectal adenomas and carcinomas. However, there is ongoing debate regarding the endoscopic examination best suited for screening purposes (Ransohoff, 2005). In particular, whilst there seems to be consensus that screening by both sigmoidoscopy and colonoscopy is cost effective, results on the relative effectiveness and cost-effectiveness of sigmoidoscopy compared with colonoscopy have not been consistent.

An assumption made in the most important previous studies is that colonoscopy, with endoscopic removal by polypectomy, is equally effective for the reduction in mortality from proximal and distal CRC. However, risk reduction may be different for both locations of CRC for several reasons, including incompleteness of colonoscopy or suggested differences in mechanisms of tumorigenesis (Lindblom, 2001).

MOLECULAR MARKERS AND THEIR COST-BENEFITS

In recent years new tests based on the molecular analysis of fecal DNA and free circulating DNA (FC-DNA) in blood have been proposed as promising tools for the early diagnosis of CRC. A noninvasive diagnostic molecular test would prove useful in identifying cancerous lesions in screening programs, and would also help to reduce the frequency of follow-up surveillance colonoscopies that are required for patients who have an increased risk of CRC development, such as those with a family history of CRC or those exposed to carcinogenic agents.

Molecular testing could thus provide user-friendly alternatives to conventional diagnostic methods. In fact, it would seem to be particularly advantageous in terms of achievable compliance rates and practicability. Therefore, several new tests based on molecular markers and aimed at detecting neoplastic cells or cell products in stool have been developed and are being evaluated at present. A variety of genetic and epigenetic alterations of oncogenes and tumor suppressor genes, together with microsatellite instability, have been investigated (Ahlquist *et al*., 2000; Calistri *et al*., 2003; Osborn and Ahlquist, 2005; Itzkowitz *et al*., 2007), with interesting results in terms of sensitivity and specificity.

Unfortunately, none of these methods can be easily transferred to clinical practice for early diagnosis programs because they are time-consuming and expensive. Using the Markov model, Song *et al.* (2004) calculated that a molecular test with a cost-effectiveness comparable to colonoscopy would cost no more than $195. A screening interval of 2 years, a sensitivity of 65% for CRCs and 40% for large polyps, and 95% specificity were assumed for the test. However, the cost of the only currently commercially available molecular test, PreGen-Plus, is much higher than that indicated by Song *et al*., 2004; Davies *et al*., 2005; Imperiale *et al*., 2004.

The discovery that tumor FC-DNA can be detected in the blood of CRC patients held out the promise of a non-invasive test for cancer. In terms of FC-DNA quantification, existing studies vary with regard to techniques used to standardization and analysis procedures in addition to, preventing a comparison of data across studies. Two studies by the same group reported plasma DNA concentrations ~ 10/15-fold higher in similar patient populations using the DipStick method with respect to those obtained using quantitative PCR (Sozzi *et al*., 2001, 2003).

A simple, rapid and relatively inexpensive assay, such as FC-DNA measurement with real-time PCR, is desirable for the purpose of early cancer detection in mass screening programs. Unfortunately, there is considerable variation in reported results, with rarely comparable estimates of mean or median FC-DNA levels. For this reason, greater effort must now be put into developing a molecular approach that is capable of detecting CRC with a high accuracy, at a cost that would permit its rapid transfer to screening programs (Zou *et al*., 2006; Itzkowitz *et al*., 2007; Umetani *et al*., 2006b; Flamini *et al*., 2006).

FREE CIRCULATING DNA IN BLOOD

The presence of DNA molecules in cell-free human plasma or serum was reported for the first time by Mandel and Metais

(1948). Using a simple perchloric acid precipitation method, they showed the presence of nucleic acids in the blood of healthy controls as well as in patients with various diseases. More recently, these observations were confirmed by other authors who showed an increased serum DNA level in patients with proinflammatory diseases such as hepatitis and systemic lupus erythematosus, in elderly patients with chronic or acute illnesses, and in individuals after exhaustive exercise. Moreover, extracellular DNA has also been isolated from lymphatic and peritoneal fluids, urine, prostatic fluids, ascites, gastric and biliary juices, sputum and stool samples (Fleischhacker and Schmidt, 2007).

Although the presence of abnormally high levels of FC-DNA in plasma or serum of cancer patients was demonstrated for the first time in 1977 (Leon *et al*., 1977), its cellular origin was determined only ~ 12 years later when Stroun *et al*. (1989) showed that FC-DNA was also derived from tumor cells. In fact, the detection of identical mutations in proto-oncogenes and tumor suppressor genes, such as *KRAS2* and *TP53*, within the tumor and in the FC-DNA of the same patient was proof of the tumor origin of FC-DNA. Moreover, the isolated material found was shown to be double-stranded DNA, mainly of low molecular weight, resistant to RNase and pronase, but digestible with DNase I. Further studies conducted in the 1990s showed that oncogene mutations, loss of heterozygosity and microsatellite shifts can be found in FC-DNA matching those occurring in a wide variety of primary tumors such as colorectal, lung, breast, uterine cervix, ovarian, bladder, pancreatic, head and neck, and liver cancers (Gormally *et al*., 2007).

More recently, it has been demonstrated that free circulating DNA may not originate from tumor and normal cells and that, in cancer patients, the nontumoral fraction derives mainly from cells surrounding the tumor tissue. The percentage of free DNA originating from tumor cells varies from one patient to another. Using a quantitative PCR approach, it has been seen that the proportion of tumor-derived free circulating DNA varies from 3% to 93% of the total circulating DNA, with the highest percentage in patients with a low overall FC-DNA level. This suggests that FC-DNA may be correlated with the stage of tumor progression (Jahr *et al*., 2001).

Although an increase in the overall level of FC-DNA and the occurrence of alterations in FC-DNA would not seem to be restricted to a specific tumor type, site, or grade, higher levels have been detected in patients with advanced disease. In fact, the percentage of adenomatous polyposis coli (*APC*) gene alterations detected in circulating DNA seems to be higher in patients with metastases than in those without metastatic disease (Diehl *et al*., 2005).

However, although tumors would appear to determine an increase in free circulating DNA, the amount present and its composition varies among patients. Fragments ranging in size from 0.2 Kb to more than 30 Kb have been seen using electron microscopy and electrophoresis on low percentage agarose gels. Moreover, DNA released from cells can be found in the form of a nucleoprotein complex (Gormally *et al*., 2007; Fleischhacker and Schmidt, 2007).

The finding that tumors shed DNA into the bloodstream has opened up new possibilities in the areas of diagnosis and prognosis, providing a surrogate source of

tumor DNA for molecular analysis in cancer and pre-cancer patients. In particular, in a multistage process such as carcinogenesis, FC-DNA may be useful as a biomarker at several phases, including the late stage of mutagenesis, clonal expansion, early detection of pre-neoplastic lesions and cancer monitoring.

Biological Mechanisms of FC-DNA Release

In healthy individuals, it is assumed that circulating DNA originates from lymphocytes or from other nucleated cells, but its origin in malignancies is not completely known. Some mechanisms have been proposed that might explain the release of DNA into the bloodstream by the tumor-host. The process of apoptosis has been advanced as a possible source of FC-DNA on the basis that FC-DNA is often characterized by an apoptotic ladder, a typical pattern of DNA fragmentation that results in small and uniform fragments of ~ 185–200 bp produced by an internucleosome cleavage of genomic DNA (Jahr *et al*., 2001).

Another potentially involved mechanism is that of cell necrosis, as higher amounts of FC-DNA have been found in patients with large or metastatic tumors (Jahr *et al*., 2001; Diehl *et al*., 2005). In particular, Jahr *et al*. (2001) induced apoptosis and necrosis in *in vitro* cultured cells and demonstrated an increase in DNA released after treatment. The DNA manifested as a "DNA ladder", which is typical of apoptosis, or as DNA with a high molecular weight, which is indicative of necrosis. Jahr *et al*. (2001) assumed that, when a tumor increases in size, vascularization becomes a problem, causing hypoxia in regions remote from blood vessels. Hypoxia induces cell death by apoptosis of tumor and nontumor cells in infiltrated tissues, just as cells may die by necrosis. Dead cells are then usually removed by phagocytes, but this digestion process may not be completely efficient and a fraction of the released soluble chromatin fragments may escape, determining a release of DNA molecules into the bloodstream.

However, it should also be noted that apoptosis is a mechanism supposedly lost by proliferating cancer cells, and great efforts are required to restore programmed cell death in malignant cells. In contrast, cell death in normal tissues occurs mainly through apoptosis. Moreover, the paradigm of apoptosis implies that epithelial cells and macrophages eliminate DNA-containing apoptotic bodies *in situ* without generating any inflammatory response (Pathak *et al*., 2006). Thus, the contribution of apoptosis to the release of tumor FC-DNA is still unclear.

Macrophages seem to play an intermediate role in the release of extracellular DNA when these cells are co-cultured with apoptotic or necrotic cells. In fact, Choi *et al*. (2005) reported that a dose-dependent increase in DNA was released into the medium when macrophages engulfed necrotic cells, while the coculture of apoptotic cells with macrophages caused a decrease in the amount of released DNA.

Diehl *et al*. (2005) showed that mutant sequences were enriched in small DNA fragments, whereas larger fragments tended to be wild type. As necrosis involves the killing of neoplastic cells and surrounding stromal and inflammatory cells within the tumor, the DNA released from necrotic regions is likely

to contain wild type DNA sequences as well as mutant sequences. Taking into consideration the findings by Choi *et al*. (2005), Diehl *et al*. (2005) hypothesized that the mutant DNA fragments found in the circulation were derived from necrotic cells that had been engulfed by macrophages. Based on these observations, levels of mutant FC-DNA should increase proportionately to rising levels of tumor necrosis.

The lysis of cancer cells shed into the circulation by micrometastases has also been advanced as a possible source of FC-DNA. Intact cells have been found in the blood of cancer patients, including those with breast, lung, and colorectal tumors. Various techniques, such as immunocytology, flow cytometry, and RT-PCR are used to detect circulating tumor cells in blood. These methods can achieve high sensitivity with one cancer cell in 10^6 blood mononuclear cells being detected (Mercatali *et al*., 2006). However, the number of circulating tumor cells does not usually correlate with the total amount of free DNA observed. Some authors have demonstrated that, for the amount of FC-DNA found, there would need to be 1,000 to 10,000 cancer cells/ml, which is far more than the highest number of cells ever isolated by current techniques (Pathak *et al*., 2006).

Another possible explanation concerns the spontaneous, active release of DNA into the blood in the form of a nucleoprotein complex. This hypothesis is based on the observation that newly synthesized, double stranded DNA appears in the medium of cultured cells, either dividing or not. Other experiments also suggest that the level of DNA release into the medium is not affected by cell turnover (Gormally *et al*., 2007).

Some authors evaluated serum DNase activity, which degrades DNA, to explore the potential mechanisms of DNA release and found that it is lower in cancer patients than in healthy donors. In fact, the increased concentration of FC-DNA in patients with colon or stomach cancer was accompanied by a decrease in DNase activity (Tamkovich *et al*., 2006). Moreover, when necrosis, angiogenesis and proliferation features in primary tumor samples were investigated, a significant association between amounts of FC-DNA present and microvessel density was found. These data suggest a link between free circulating DNA and tumor angiogenic status (Sozzi *et al*., 2003).

Finally, there is no clear evidence whether FC-DNA release has a biological significance. Free DNA molecules may reenter cells and work as transfected gene fragments to modify the genetic make-up of host cells. The mechanisms that are responsible for a horizontal gene transfer are not clear, but apoptosis may be involved (Halicka *et al*., 2000). The authors of some studies on animal models introduced the "genometastasis hypothesis", suggesting that FC-DNA may play a role in the progression of tumors and in the development of metastases by the horizontal transfer of tumor DNA sequences with transforming potential. This hypothesis would seem to indicate an alternative pathway to explain the development of distal metastases. In a recent report, the same group also reported that, although apoptotic bodies appear to be related to cancer because they are found in increased quantities in serum, they would not seem to be the vehicles for tumor-derived free DNA detected in the early stages of cancer and during tumor progression (Samos *et al*., 2006).

Methodological Aspects of FC-DNA

Sources and Influence of Pre-analytical Factors

FC-DNA is usually isolated from plasma or serum, and several studies have shown that the concentration of FC-DNA is four- to tenfold lower in the former than in the latter. The existence of coagulation factors and their related proteins, in addition to platelets in plasma, is probably the most significant cause of the difference between these two sources. Furthermore, the finding that serum DNA concentration correlates with leukocyte counts suggests that the leukocytes ruptured during serum separation may release DNA, causing an increase in free DNA, and thus explaining why it is more abundant in serum than in plasma. If free DNA is released during purification, FC-DNA obtained from serum would, in theory, lead to more erroneous results due to extraneous DNA derived from leukocytes. However, these observations have yet to be confirmed (Gautschi *et al*., 2004).

It has also been hypothesized that an unequal distribution of DNA during separation from whole blood might be the cause of different levels in serum and plasma. If that were the case, the use of free DNA derived from serum would increase the sensitivity of FC-DNA analysis. To verify this hypothesis, the amount of DNA in serum and plasma concurrently separated from the same blood sample within 6 h of withdrawal and under the same conditions was determined by Umetani *et al*. (2006a). FC-DNA was quantified by real-time quantitative PCR for the ALU repeats, which is the most abundant repeat sequence (1.4×10^6 copies) in the human genome. ALU-qPCR was sensitive enough to allow the use of minimally processed serum and plasma without DNA purification. Results showed that the lower level of DNA in plasma was not due to DNA loss during purification of DNA from plasma. A significant positive regression was also shown between DNA in serum and in plasma; the estimated quantity of extraneous DNA in serum was only 8.2% of the total serum DNA. It was also seen that the excess level of DNA in serum was not principally derived from DNA released from leukocytes or other sources during the separation of serum. A possible explanation for the difference in serum and plasma DNA levels could be unequal distribution of DNA during separation from whole blood. Based on the estimated scale factor of serum DNA in relation to plasma DNA, serum contains approximately six times as much FC-DNA as plasma, suggesting that serum is a better source for circulating cancer-related DNA detection.

However, the problem of cellular DNA contamination still remains, and it has been estimated that the amount of contamination increases as the time interval between blood withdrawal and serum isolation increases (Gormally *et al*., 2007). Fleischhacker and Schmidt (2007) reported that there was no change in extracellular DNA concentration when EDTA-stabilized blood samples were stored for 8 h at room temperature or for 24 h at 4°C.

Measuring FC-DNA concentrations in the plasma of healthy donors recruited from 23 different European centers, Gormally *et al*. (2004) observed that a strong variation in plasma FC-DNA concentration existed between recruitment centers ($P < 0.0001$). The different protocols used for blood processing and, in particular, the time between blood drawing

and separation may help to explain this large variation. In fact, the rapid processing of plasma or serum is crucial before storage at −20°C or lower.

The best conditions for cryopreservation of FC-DNA have not been extensively analyzed. It has been demonstrated that it is possible to identify K-ras mutations in plasma DNA after more than 6 years of storage (Kopreski *et al*., 1997), but it has also been seen that the amount of FC-DNA decreases by a factor of 0.66 genome equivalent/ml per month of storage (Lee *et al*., 2002), showing a gradual reduction in sensitivity over time. This data was recently confirmed, demonstrating that 30% of DNA was degraded annually in plasma stored at −80°C or in isolated DNA stored at −20°C (Sozzi *et al*., 2005). In a genotyping study, it was found that archival plasma stored for up to 25 years could still be used to extract DNA of sufficient quality for PCR analysis (Sjöholm *et al*., 2005), confirming that the development of a standardized analysis protocol is still an important issue.

Isolation and Quantification

The quantity of extracellular DNA that can be isolated from human plasma and serum is frequently low and of poor quality. A standardized method for the isolation of FC-DNA does not exist and the protocols designed for this purpose are probably as numerous as the laboratories using them. FC-DNA can be extracted by phenol/chloroform or by commercially available columns based on ion-exchange binding of DNA. Although these kits facilitate rapid DNA isolation, part of the DNA is lost as the columns are only effective in binding nucleic acid molecules > 100–150 bp. On the other hand, when a very crude extraction method is used, almost 50% of the PCR reactions fail, even when high DNA concentrations are measured (Fleischhacker and Schmidt, 2007).

There is also no standard method for DNA quantification. In early studies, DNA was quantified directly by colorimetric or fluorometric assays using reagents which, when added to the plasma or serum, produced a color change, the degree of which correlated with the DNA concentration. The use of these assays in quantitative analyses is limited because of their poor specificity and sensitivity.

Nanogram concentrations of FC-DNA can be measured more efficiently by DNA hybridization, radioimmunoassay or nick translation assays. The DNA DipStick TM kit (Invitrogen, Carlsbad, CA) has been shown to detect between 0.1 and 10 ng of free DNA (Sozzi *et al*., 2001). The detection of nucleosomes by immunoassays (ELISA) is an alternative method to assess DNA concentrations, but the results generated with these methods, expressed in arbitrary units, do not allow a comparison with other tests. More recently, the use of real-time PCR and PicoGreen double-stranded DNA quantification assays has also made it possible to quantify picogram amounts of FC-DNA (Flamini *et al*., 2006; Chang *et al*., 2002), providing a more accurate and sensitive analysis of free circulating DNA.

FC-DNA Levels in Colorectal Tumor Cancer Diagnosis

The differences observed in FC-DNA concentrations between cancer patients and healthy subjects have led to the hypothesis that FC-DNA levels could be a potentially useful tool for early cancer

detection. In one of the first studies carried out in this area ~ 20 years ago, Shapiro *et al.* (1983) detected FC-DNA in the serum of patients with benign or malignant gastrointestinal disease. FC-DNA was quantified by radioimmunoassay capable of detecting 25 ng/ml of FC-DNA. Patients with benign gastrointestinal disease had significantly higher DNA levels than controls ($P < 0.001$). A comparison between patients with malignant or benign colorectal lesions showed that the former had significantly higher DNA levels ($P < 0.05$). These authors also compared serum levels of FC-DNA and carcinoembryonic antigen (CEA) to assess the diagnostic potential of simultaneous determinations of markers associated with neoplastic disease. In particular, sensitivity increased fairly dramatically in the subgroup of CRC patients, increasing from 55% for DNA and 69% for CEA to 90% for both markers, while specificity decreased only slightly (from 79% to 72%).

In a large clinical study including different neoplastic and non-neoplastic patients and healthy volunteers, it was seen that there was a highly statistically significant difference in average FC-DNA levels between cancer patients and both healthy controls and patients with non-neoplastic disease. However, no cut-off value for FC-DNA concentration produced performance characteristics that would make it a good screening tool for neoplastic disease (Chang *et al*., 2002).

In his study on CRC patients, Thijssen *et al*. (2002) demonstrated that serum and plasma FC-DNA levels did not correlate and that each value had a different correlation with diagnosis and prognosis. Whilst serum DNA concentration was significantly associated with the presence of liver metastases, plasma DNA was only predictive of disease recurrence.

Recently, Umetani *et al*. (2006b) developed a novel method with high sensitivity to measure the ratio of longer to shorter DNA fragments (DNA integrity) in serum as a potential biomarker for patients with CRC and periampullary cancers. Sera from 51 healthy volunteers, 32 patients with CRC and 19 patients with periampullary tumors were assessed by quantitative PCR. Two sets of primers were used for ALU repeat amplification. The primer set for the 115-bp amplicon (ALU115) amplifies both shorter and longer DNA fragments (absolute amount of DNA), whereas the primer set for 247-bp amplicon (ALU247) amplifies only longer DNA fragments. DNA integrity was calculated as the ratio of quantitative PCR results of 247-bp over 115-bp fragments. The absolute equivalent amount of DNA in each sample was determined using a calibration curve with serial dilutions (10 ng to 0.01 pg) of genomic DNA obtained from the peripheral blood of healthy volunteers. Results were expressed in nanograms per microliter and DNA integrity was calculated as the ratio of quantitative PCR results with two primer sets. For CRC patients, the area under the receiver operating characteristic (ROC) curve to distinguish patients from healthy controls by absolute DNA concentrations was 0.75. The ROC curve for serum DNA integrity was 0.78. Mean absolute serum DNA concentrations in patients with stage I/II and stage III/IV CRC were 1.63 and 1.73 ng/μl, respectively, which were significantly higher than in healthy volunteers. The mean serum DNA integrity in patients with stage I/II and stage III/IV CRC was 0.22 in both subgroups, which was significantly higher than in healthy

volunteers. The combined index of absolute concentration and integrity of serum DNA achieved 92% specificity and 63% sensitivity for DNA detection in the two patient populations.

Another recent study proposed a quantitative approach using the DipStick Kit method to measure FC-DNA in plasma (Frattini *et al*., 2006). Results were expressed as nanograms per milliliter. The prognostic study was carried out on 20 healthy donors and 70 patients with CRC. In patients, FC-DNA levels were 25-fold higher than those of healthy donors. The mean value of FC-DNA was 10.3 ng/ml in healthy subjects and 495.7 ng/ml in CRC patients. In the same cohort, the CEA value was altered in only 26 cases (37%), a finding in keeping with the literature data.

Flamini *et al*. (2006) determined FC-DNA in serum using a simple, rapid and relatively inexpensive test in a case-control study carried out on 75 healthy donors and 75 CRC patients. To quantify FC-DNA, the *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* housekeeping gene was amplified by a real-time quantitative PCR assay using SYBR Green I Dye Chemistry and MyIQ Single-Color Real-time PCR Detection System (Bio-Rad, Hercules, CA). The PCR mix was prepared in a total volume of 25 µl containing 1x Sybr Green Supermix (Bio-Rad), 0.4 µmol/l of each primer, and 5 µl of DNA. The absolute concentration of target DNA was calculated on a standard curve using concentrations ranging from 25 to 0.01 ng of DNA extracted from the peripheral blood of a healthy donor, and results were expressed as nanograms per milliliter. In the absence of internationally available cut-off values for serum-free DNA, the cut-off maximally discriminating between healthy donors and patients was identified using ROC curve analysis. FC-DNA levels were fivefold higher in the serum of patients than in that of controls and were not related to either age or gender. Median levels were already high in patients with early-stage tumors. The test showed good diagnostic accuracy, with sensitivity ranging from 71% to 91% and specificity varying from 53% to 93% for the cut-offs of 8.5, 12.5, 16.5, and 20.5 ng/ml. When 12.5 ng/ml was used as the cut-off value, 81% sensitivity and 73% specificity were observed for the overall series. More importantly, when FC-DNA was considered in combination with the more conventional CEA, sensitivity increased to 88%, showing that FC-DNA and CEA provided independent diagnostic information. ROC curve analysis of the combined FC-DNA and CEA algorithms showed a higher diagnostic capacity (area under the ROC curve, 0.92) than that of each single marker. Furthermore, the best cut-off point of this algorithm was associated with 84% sensitivity and 88% specificity, indicating that the combination of the two markers could be a useful tool for the diagnosis of early-stage disease.

Although a variety of different methods have been used to estimate DNA concentrations in blood, real-time PCR is currently the most widely employed method in laboratories. The simple measurement of FC-DNA using quantitative PCR could offer a cost-effective approach for population-based CRC screening, even though results are not often comparable due to the different genes analyzed, such as *GAPDH*, β-actin or ALU repeats (Flamini *et al*., 2006; Wang *et al*., 2003; Umetani *et al*., 2006a). Moreover, there is little consensus on how samples should be collected,

stored, or processed, and there is no universal answer to the question as to whether serum or plasma is better suited for the analysis of FC-DNA. Generally, higher concentrations of FC-DNA are obtained in serum than in plasma, and a recent study showed that this is not caused by contaminated extraneous DNA during separation (Umetani *et al*., 2006a). For these reasons, further studies should use a standardized methodology and large, clearly defined patient and control populations in order to obtain more interpretable results.

EVALUATION OF DNA INTEGRITY OF GENOMIC DNA EXTRACTED FROM STOOL

Analysis of Long Fragment DNA in Stool

DNA amplification of exfoliated cells in stool represents another moderately inexpensive and relatively rapid test for the early diagnosis of CRC. Preliminary evidence (Ahlquist *et al*., 2000) has shown that the evaluation of DNA amplification of some DNA fragments longer than 200 bp (long DNA or L-DNA) detects more than 50% of colorectal cancers, with very high specificity. In fact, it has been observed that genomic DNA extracted from stool is more easily amplifiable than that obtained from healthy individuals, suggesting that better conserved DNA is found in the stool of patients with CRC. An epigenetic phenomenon may be responsible for this in that there is a more abundant exfoliation of nonapoptotic cancer cells (Ahlquist *et al*., 2000), mainly due to the cancer fraction, in patients with CRC. In contrast, cells shed from normal mucosa are largely apoptotic, and endonucleases activated by the apoptotic process lead to the formation of "short" DNA molecules.

This preliminary result was extensively analyzed in a recent multicenter study (Imperiale *et al*., 2004), which did not, however, confirm the preliminary results, obtaining poorer sensitivity in CRC detection. This may be because the method does not give a real quantification of long fecal DNA and thus does not permit an accurate continuous-scale analysis of the best cut-offs capable of discriminating between colorectal patients and healthy individuals.

To overcome this problem, a quantitative approach called "fluorescence long DNA analysis" (FL-DNA) was developed. This method was validated in a pilot case-control study (Calistri *et al*., 2004), and appears to be capable of accurately detecting more than two thirds of patients with CRC. This approach involves of the extraction of genomic DNA from stool and quantification, by fluorescence analysis, of DNA fragments longer than 200 bp.

A small amount of stool is homogenized and, after centrifugation, all particulate matter is removed. The DNA contained in the supernatant is recovered by precipitation and then purified by a commercial kit (QIAamp DNA Stool Kit, Qiagen, Hilden, Germany). To evaluate DNA status, the fluorescence intensity of eight sample-specific PCR products is determined using a fluorescent-labelled primer (Calistri *et al*., 2003, 2004). This amplification product is designed to amplify *p53* or *APC* gene fragments between 200 and 400 bp in length. DNA from each sample is quantified on the basis of a standard curve (1, 2, 5, 10, and 20 ng) of genomic DNA and results are expressed as nanograms.

The analysis is carried out on 2 µl of stool DNA and the amplification products

are quantified by electrophoresis analysis using a capillary electrophoresis apparatus (3,100 Avant Genetic Analyzer, Applied Biosystems, Foster City, CA) equipped with specific software to determine the amount of fluorescence signal of PCR products (GeneScan Analysis 3.7). To verify the presence or absence of Taq inhibitors, amplification with a mix containing a plasmid with a control sequence is carried out in all samples.

A case-control study was carried out by Calistri *et al*. (2004) using this quantitative test to validate its capacity to detect CRC. ROC curve analysis of FL-DNA levels was used to determine the best cut-offs capable of accurately discriminating between colorectal tumors and healthy individuals. The fluorescence method showed good diagnostic accuracy, with specificity ranging from 83% to 95% and sensitivity ranging from 82% to 72% for the cut-offs of 15, 20, 25, and 30 ng of DNA.

When the cut-off of 25 ng, which provided the best overall accuracy, was analyzed in relation to different tumor characteristics, sensitivity remained high in patients independently of tumor size or Dukes' stage. More importantly, similar sensitivity was observed in detecting tumors localized in ascending and descending colon tracts, indicating the possibility of evaluating the entire intestinal tract with the same efficiency. These results were recently validated in a confirmatory study (unpublished data), indicating the potential usefulness of this test in screening programs or in monitoring members of families at risk for CRC.

More recently, another quantitative approach based on the evaluation of ALU sequences was proposed (Zou *et al*., 2006). Genomic DNA stool extraction was performed as previously described using a commercial kit (QIAamp DNA Stool Kit, Qiagen) but differing in terms of the molecular target and the quantification approach. In fact, stool DNA integrity was quantified by amplifying a 245-bp fragment within ALU repeats and by real-time analysis with SYBR Green fluorescence. Whilst the method showed slightly lower sensitivity than that observed with the other quantitative approach proposed, it presented advantages in terms of simplicity and speed of execution. However, further evaluation is needed in larger case-control studies along with using a larger panel of DNA fragments.

The importance of this marker and the interest shown in this new approach have also been demonstrated in studies carried out combining DNA integrity with other molecular targets. Furthermore, Itzkowitz *et al*. (2007) recently reported on a new generation of commercial molecular tests for the early diagnosis of CRC presenting this characteristic. Although the study, based on an evaluation of a combination of gene mutations, epigenetic alterations and analysis of fecal genomic DNA integrity, indicated that the association of different molecular alterations could be used to increase sensitivity to detect tumors, no molecular marker has, to date, presented the same high accuracy as fecal long DNA analysis (Itzkowitz *et al*., 2007).

Advantages and Technical Problems

The advantages of the long DNA test, as indicated in the previous paragraphs, lie mainly in the possibility of performing the analysis with small amounts of stool, similar to the amount used for other noninvasive approaches such as FOBT, and the fact that

there is no need for a specific diet or bowel preparation. Moreover, the use of molecular targets could provide a more specific and target-related analysis that could overcome problems of false-positive results, a frequent occurrence when using biological targets not necessarily related to tumor or premalignant lesions such as blood in feces or CEA analysis in peripheral blood. Current results are obviously not exhaustive, and other targets and molecular approaches probably need to be studied.

The main technical problem of this approach and possibly of all the fecal DNA alteration analyses is DNA conservation (Zou *et al*., 2006).

Experimental observations confirm the instability of human long DNA during prolonged preanalysis stool storage, probably caused by bacterial DNases present in fecal samples (Zou *et al*., 2006). Whilst this problem could theoretically be overcome by correct conservation and by the use of buffers containing EDTA, attention must also be paid to incorporating a DNase inhibitor as part of specimen collection and processing. Moreover, DNA extracted from stool could present Taq inhibitors that interfere with PCR analysis. This problem could be resolved by performing DNA extraction with a specific buffer, using a purification procedure capable of removing these inhibitors, and then verifying their absence using an internal control (Calistri *et al*., 2004).

CONCLUSIONS

Many authors have suggested that cancer screening, independently of the methodological approach used, must be cost-effective in the long term (Davies *et al*., 2005). However, it must also be remembered that the choice of a particular screening protocol is based on a number of important considerations, such as costs, availability of an adequate laboratory, patient compliance and physician specialization. All these aspects were analyzed according to the Markov model in a study by Song *et al*. (2004). Results showed that a DNA-based test capable of detecting approximately two thirds of CRC patients with low sensitivity for large polyps and high specificity for colorectal lesions would be a valid cost-effective alternative to the current gold-standard, colonoscopy, if the cost of this test were no more than $195.

As already discussed, numerous DNA assays have been investigated in the past few years and a commercial multitargeted test based on the most interesting molecular targets has been developed (Davies *et al*., 2005; Imperiale *et al*., 2004). This approach shows good accuracy, with higher sensitivity than the Hemoccult II test and similar specificity (Imperiale *et al*., 2004). Unfortunately, its high cost, calculated from price indications provided by the Markov model, suggests that this test cannot be considered a valid alternative to current diagnostic methods (Song *et al*., 2004).

In contrast, the tests based on the evaluation of DNA status in biological fluids seem to be perfectly aligned with Markov model prerequisites. They are, in fact, capable of identifying a high percentage of CRCs and present a much lower unit cost than that established in the Song *et al*. (2004) analysis. Furthermore, the tests are relatively simple to perform and the development of a kit would probably help to further reduce costs and execution time, making them valid alternatives to approaches currently used in CRC screening programs.

Obviously, the extension of these tests, as of any others, to screening programs means that many open questions must be addressed and resolved. A test capable of detecting all adenomas might thus not be the best approach for early CRC diagnosis especially if we consider that only a small fraction of adenomas actually progress into cancers. In fact, such an approach would probably lead to overtreatment in the vast majority of cases, which would be costly and potentially harmful, both in physical and psychological terms (Haug and Brenner, 2005). An important task now for researchers is to identify which molecular markers are determinants of malignant progression (Davies *et al*., 2005). This would not only facilitate the clinical management of CRC but would also help us to understand which premalignant lesions require early detection through screening programs in order to reduce mortality risk. With regard to FC-DNA and DNA fecal tests, the next step will most probably involve verifying their predictive accuracy for CRC in screening programs alongside current standard diagnostic procedures, FOBT and colonoscopy, to identify pre-malignant and malignant lesions. Molecular tests that are noninvasive, relatively simple to perform, require a small amount of blood or stool and show high diagnostic accuracy would appear to be valid tools to use in combination with or as an alternative to current approaches used for CRC diagnosis.

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6
Colorectal Carcinoma: Identification of MicroRNAs Using Real-Time Polymerase Chain Reaction

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INTRODUCTION

Cancer is fundamentally a genetic and epigenetic disease requiring the accumulation of genomic alterations that inactivate tumor suppressors and activate protooncogenes. Classical tumor suppressors such as retinoblastoma1 (*RB1*) and *TP53* and oncogenes such as *MYC* and *RAS* have been extensively studied and found to be involved in complicated interacting pathways that regulate cell-cycle progression and apoptosis.

Recently, the classical family of protein-coding genes recognized as tumor suppressors and oncogenes has been expanded to include a species of RNA molecules known as microRNAs (miRNAs). miRNAs are 18–24-nucleotide RNA molecules that base-pair with target mRNAs and negatively regulate their stability and translational efficiency. The first evidence that these molecules play a role in cancer pathogenesis came from studies in model systems where it was demonstrated that specific miRNAs contribute to the regulation of cellular differentiation, proliferation, and apoptosis. Consistent with a role in controlling these processes, numerous studies have now documented widespread dysregulation of these molecules in diverse cancers. Although relatively few studies have dissected the causative role of specific miRNAs in tumorigenesis, the available evidence suggests that miRNAs function in concert with classical tumor suppressors and oncoproteins to regulate key pathways involved in cellular growth control.

Colorectal cancer is one of the major causes of cancer death worldwide. At a molecular level, much progress has been made in the last 2 decades in the identification and characterization of the genetic changes involved in the malignant colorectal transformation process. The multistep carcinogenesis model described by Fearon and Vogelstein (1990) in colon cancer serves as the classical model of genetic alterations in cancer. A number of molecular studies have shown that colon carcinogenesis results from an accumulation of epigenetic and genetic alterations, including activating mutations of the *K-RAS* protooncogene and inactivating mutations of *APC* and *TP53* tumor suppressor genes or of DNA repair genes. However, this stepwise model of colorectal tumorigenesis has been mainly validated conceptually, and there is increasing evidence that alternative genetic events may occur during colorectal carcinogenesis, sometimes preferentially, sometimes randomly, and sometimes with an overlap.

The ability to effectively profile miRNA expression could lead to the discoveries of disease-specific miRNA biomarkers, as well as contribute to an understanding of how miRNAs regulate cancer cells. In colorectal cancer, miRNA expression regulation could help to identify mRNA targets associated with different colorectal carcinogenesis pathways and their role as potential therapeutic targets. While the miRNA field is still emerging, the benefit of our understanding of miRNA in cancer is potentially enormous, especially if we are able to apply this knowledge to provide new therapies for patients.

miRNAs were first identified in 1993 when Lee *et al*. (1993) found that a 22-nucleotide RNA called lin-4 is required for the appropriate timing of postembryonic development in *Caenorhabditis elegans*. Since this discovery, miRNAs have been identified in diverse animals and plants, and it now seems likely that all multicellular eukaryotes and perhaps some unicellular eukaryotes utilize these RNAs to regulate gene expression. In humans, over 475 miRNAs have been identified, and it is predicted that the human genome encodes up to 1,000 miRNAs (Griffiths-Jones, 2006).

miRNA BIOGENESIS AND FUNCTION

miRNA genes are scattered in all chromosomes in humans except for the Y chromosome. Approximately 50% of known miRNAs are found in clusters, and they are transcribed as polycistronic primary transcripts. The miRNAs in a given cluster are often related to each other, suggesting that the gene cluster is a result of gene duplication. A miRNA gene cluster also often contains unrelated miRNAs. A plausible but yet-to-be validated possibility is that the clustered miRNAs are functionally related by virtue of targeting the same gene or diff-erent genes in the same pathway. It was initially thought that most miRNA genes were located in intergenic regions. However, recent analyses of miRNA gene locations showed that the majority (70%) of mammalian miRNA genes are located in defined transcription units (TUs). Rodriguez *et al*. (2004) demonstrated that many miRNA genes were found in the introns in the sense orientation, which is more than previously expected. Of these intronic miRNAs, the majority of miRNAs are in the introns of protein-coding genes, whereas a lesser number of miRNAs are in the introns of noncoding RNAs (ncRNAs). This indicates that previous informatic searches confined to intergenic regions might have missed some miRNA genes. The location of some intronic miRNAs is well conserved among diverse species. In some exceptional cases, miRNAs are present in either an exon or an intron ('mixed'), depending on the alternative splicing pattern.

miRNAs are transcribed by RNA polymerase II to produce a primary-miRNA (pri-miRNA). Pri-miRNAs are long nucleotide sequences usually capped at the 5′-end and polyadenylated at the 3′-end regions. Then, pri-miRNAs form specific hairpin-shaped stem-loop secondary structures and are cleaved by the nuclear RNase III Drosha to release the precursor of miRNA (pre-miRNA). Drosha requires a cofactor, the Disgorge syndrome critical region gene 8 (DGCR8), or Pasha. The pre-miRNAs are then transported to the cytoplasm by Exportin-5 (Exp5). Once in the cytoplasm, pre-miRNAs are further processed to a short double-strand miRNA (18–22 nucleotides) by Dicer, a second RNase III endonuclease. Mature miRNAs are incorporated into the effector complex known as miRISC (miRNA-containing RNA-induced silencing complex). During

RISC assembly, the cleavage products are rapidly converted into single strands. Usually one strand disappears, whereas the other remains as a mature miRNA, and it incorporates into a ribonucleotide complex that carries out its function of silencing gene expression.

Although the exact silencing mechanism is unknown, it is clear that miRNAs use a mode of silencing similar to that employed by siRNAs, which cleave mRNA transcripts. The mechanism of regulation depends on the degree of complementarity between a miRNA and its target. When a miRNA and an mRNA exhibits perfect complementarity, the target mRNA is cleaved by RISC. This is the predominant mechanism through which miRNA functions in plants. Imperfect base pairing between a miRNA and its target, as occurs with most mammalian miRNAs, leads to translational silencing of the target. However, imperfect complementary miRNAs can also reduce the abundance of mRNAs. The current challenge is to accurately identify biological targets that are regulated by miRNAs. Figure 6.1 illustrates miRNA biogenesis and action mechanisms.

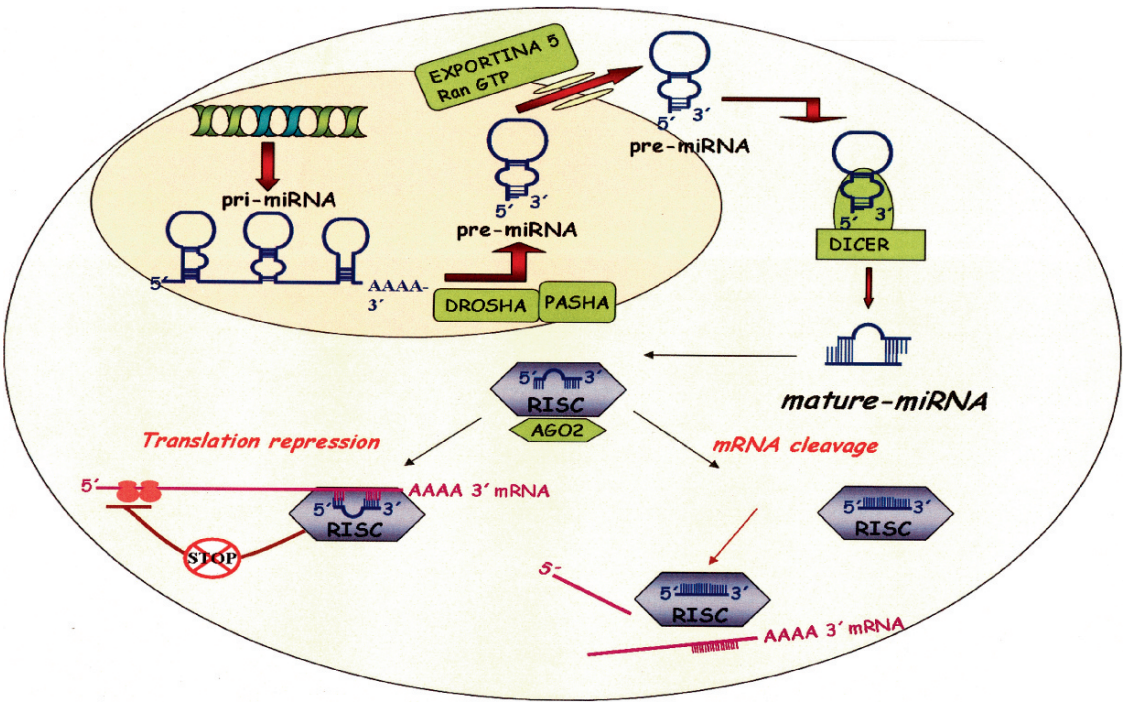

FIGURE 6.1. The biogenesis of microRNAs. MicroRNA (miRNA) genes are generally transcribed by RNA polymerase II in the nucleus to form large pri-miRNA transcripts, which are capped and polyadenylated. These pri-miRNA transcripts are processed by the RNase III enzyme Drosha and its co-factor, Pasha, to release the 70-nucleotide pre-miRNA precursor product. RAN–GTP and exportin 5 transport the pre-miRNA into the cytoplasm. Subsequently, another RNase III enzyme, Dicer, processes the pre-miRNA to generate a transient 22-nucleotide miRNA:miRNA* duplex. This duplex is then loaded into the miRNA-associated multiprotein RNA-induced silencing complex (miRISC), which includes the Argonaute proteins. The mature miRNA then binds to complementary sites in the mRNA target to negatively regulate gene expression in one of the two ways that depend on the degree of complementarity between the miRNA and its target. miRNAs that bind to mRNA targets with imperfect complementarity block target gene expression at the level of protein translation. miRNAs that bind to their mRNA targets with perfect complementarity induce target-mRNA cleavage

EVIDENCE FOR THE INVOLVEMENT OF miRNA IN CANCER

miRNA-associated genes have been implicated in human cancers. Karube *et al*. (2005) have shown that Dicer expression is downregulated in lung cancer and reduced expression of Dicer correlates with shortened postoperative survival. However, injection of Dicer-deficient mouse embryonic stem cells into nude mice failed to generate tumors. Dicer disruption studies in mice have shown that this gene is important during mammalian development. Argonaute proteins, clustered on chromosome 1 and components of RISC complex, are frequently deleted in Wills tumors and are associated with neuroectodermal tumors. Moreover, 50% of miRNAs are located in areas of the genome known as fragile sites, which are frequently amplified, deleted, or rearranged in cancer, suggesting that miRNA abnormalities play an important role in cancer pathogenesis.

A more direct link between miRNA function and cancer pathogenesis is supported by studies examining the expression of miRNA in clinical samples. Currently, almost all of the miRNA expression studies on cancers are based on determining the expression profile of miRNAs in cancer cells versus normal cells. Recognition of miRNAs that are differentially expressed between tumor tissues and normal tissues may help to identify those miRNAs that are involved in human cancers and further establish the apparent pathogenic role of miRNAs in cancers.

The initial evidence for the involvement of miRNAs in cancer was derived from a study in human chronic lymphocytic leukemia (CLL). Calin *et al*. (2002) reported that two miRNAs, miR-15a and miR-16a, are located in a region, 13q14, commonly deleted in B-CLL (B-chronic lymphocytic leukemia). Expression analysis indicated that miR-15 and miR-16 were either absent or downregulated in the majority (68%) of CLL patients. It was later shown that miR-15a and miR-16-1 expression silenced the anti-apoptotic factor bcl-2, suggesting that their low or absent levels in CLL inhibit apoptosis by reactivation of bcl-2. Next, the same group (Calin *et al*., 2005) reported mutation in a small group of miRNAs in some patients with CLL and identified a miRNA expression signature composed of 13 miRNAs associated with prognostic factors and disease progression in CLL.

Changes in the expression of miRNAs have been observed in a variety of human tumors. Examination of miRNA expression patterns in lung cancers identified a reduction in tumoral samples of the let-7 miRNA. Yanaihara *et al*. (2006) classify lung cancer patients into two major groups according to the let-7 expression, and showed that patients with let-7 downregulation had significantly shorter survival after surgical resection. Overexpression of let-7 was shown to inhibit cancer cell growth *in vitro*, and let-7 negatively regulated the expression of RAS and MYC by targeting their mRNA for translation repression. The correlation of let-7 levels with disease outcomes in lung cancer has been confirmed in other studies, which also implicated miR-155 as a prognostic factor. In contrast to let-7, the expression of miR-17-92 cluster was increased in lung cancer, and overexpression also enhanced lung cancer cell growth (Hayashita *et al*., 2005). For breast cancers, Iorio *et al*. (2005) have

reported a miRNA profile for distinguishing breast tumors from normal epithelium, which have been correlated with specific pathological features, such as tumor stage, proliferation index, and hormone receptor expression. miRNAs are also involved in human brain cancer. Ciafrè *et al*. (2005) observed that miR-221 was strongly upregulated in glioblastoma samples and that miR-181a, miR-181b, and miR-181c were downregulated in glioblastoma compared to normal brain samples. Another study in glioblastoma cancer conducted by Chan *et al*. (2005) identified miR-21 as overexpressed in highly malignant human glioblastoma. Eis *et al*. (2005) found that miR-155 is overexpressed in a wide range of lymphomas derived from B cells of different development stages, including Burkitt's lymphoma. Moreover, overexpression was higher in diffuse large B cell lymphoma, a more aggressive B cell neoplasm. They also found that significantly higher levels of miR-155 were observed in the cells with ABC phenotype, suggesting that this miRNA may be useful for differential diagnosis. In addition to miR-155, miR-15a was also underexpressed in diffuse large B cell lymphoma. On the other hand, miR-17-92 cluster was found to be overexpressed in many types of lymphoma samples. In human testicular germ cell tumors, miR-372 and miR-373 were found to function as oncogenes. These miRNAs were overexpressed in human testicular germ cells and neutralized p53-mediated CDK inhibition of tumoral cells through direct inhibition of the suppressor gene LAST2. Murakami *et al*. (2006) investigated the miRNA expression profile of hepatocellular carcinoma and paired nontumoral tissues and found that miR-18 and miR-224 were significantly overexpressed; miR-199a*, miR-195, miR-199a, miR-200a, and miR-125a were underexpressed in tumoral tissues compared with nontumoral tissues. Colon cancer is also associated with altered miRNA expression. Michael *et al*. (2003) discovered by cloning technique that the expression of two mature miRNAs, miR-143 and miR-145, was consistently reduced at the adenomatous and cancer stages of colorectal neoplasm. A more recent miRNA serial analysis of gene expression (miRAGE) was utilized to compare expression levels of miRNAs in two primary colorectal adenocarcinomas with matched normal colonic epithelia. Cummins *et al*. (2006) identified 50 differentially expressed miRNAs, and miR-145 and miR-143 were also significantly lower in tumoral cells compared with normal colonic cells. Later, Bandres *et al*. (2006) examined by real-time PCR the expression of 156 mature miRNAs in a panel of 16 colorectal cell lines and 12 matched-pairs of tumoral and nontumoral tissues from patients. This work identified a subset of 13 miRNAs differentially expressed in colorectal cell lines and clinical samples; among them miR-145 was also identified as downregulated in colorectal tissues. Moreover, the expression levels of miR-31 were higher in the tumor samples and colorectal cell lines in comparison with the nontumoral samples and were related to pathological stage, suggesting that this miRNA could contribute to both the tumorogenesis and the acquisition of a more aggressive phenotype in colorectal cancer. More recently, Roldo *et al*. (2006) investigated the global miRNA expression patterns in normal pancreas, pancreatic endocrine tumors, and acinar carcinomas. The data showed that the expression of miR-103 and miR-107

associated with lack of expression of miR-155 distinguishes tumoral samples from normal pancreas. Moreover, a set of ten miRNAs distinguishes endocrine from acinar tumors, and the overexpression of miR-21 is strongly associated with Ki-67 proliferation index and presence of liver metastasis.

Although expression differences may not be causal events of tumorigenesis, such changes may regulate important genes in carcinogenesis and may be useful for classifying tumors and predicting their outcomes. Perhaps, because miRNAs play a central role in development and directly affects global gene regulation, they could be considered as important markers for human cancer.

It has been suggested that tumor miRNA profile may resemble that of its antecedent stem cells, and thus reflect development lineage. In this sense, Lu *et al*. (2005) analyzed the expression levels of 217 miRNAs across 334 primary tumors, normal tissues, and cell lines, and their results showed that tumors display a miRNA expression profile reminiscent of that in the tissues from which they were derived. The miRNA profile was a better indicator of tissue lineage than the mRNA profile. Our understanding of miRNA function in mammals suggests that these molecules play a role in determination and maintenance of lineage during development.

The molecular mechanism underlying the alteration of miRNA expression in cancer is mostly unknown; hypothetically, specific transcriptional inhibition, epigenetic mechanisms of DNA methylation and histone deacetylation, mutations affecting processing and maturation, or regulation of miRNA stability could cause this disease.

In lung cancer, epigenetic mechanisms have been shown to regulate let-7 miRNA downregulation. However, treatment of A549 lung cancer cells with either of the two demethylating agents, 5-aza-dC or 5-aza-C, or either of two HDAC inhibitors, TSA or sodium butyrate, did not demonstrate significant alterations in miRNA expression patterns. These results suggest that global epigenetic silencing of miRNA expression is unlikely to underlie the reduced expression observed in these cancer cells. This observation differed from the study carried out by Scott *et al*. (2006), which suggested that treatment of a breast cancer cell line with a proapoptotic dose of the HDAC inhibitor hydroxamic acid LAQ824 resulted in both upregulation and downregulation of many miRNA transcripts, accompanying the induction of apoptosis. This discrepancy may reflect different responses in the cell lines tested as well as dose-dependent effects of the different HDAC inhibitors. In this sense, the analysis realized by Saito *et al*. (2006) of the miRNA expression profile in T24 human bladder cancer cells without treatment or treated simultaneously with 5-aza-dC and 4-phenilbutiric acid revealed that 17 of 313 miRNAs were upregulated. One of these, miR-127, is embedded in a CpG island, and its expression is regulated by methylation of its promoter in cancer cells. More recently, Lujambio *et al*. (2007) have shown that one mechanism accounting for the observed downregulation of miR-124a in colorectal cancer (CRC) cancer is CpG island hypermethylation, in a manner similar to that well accepted for classic

tumor suppressor genes. Further studies on epigenetic regulation of miRNA expression are necessary to elucidate its role as an important mechanism responsible for miRNA expression.

On the other hand, Diederichs and Haber (2006) screened 15 miRNAs linked to tumorigenesis by virtue of their mRNA targets or their chromosomal localization in 91 cancer-derived cell lines, and did not find any evidence of mutations that altered the sequence of the mature miRNA. The authors identified 16 sequence aberrations in miRNA precursors, including some with effect on their secondary structure, but none altered the ability of precursors to be processed to the mature form *in vivo*. The small size of the miRNA in comparison with the mRNAs could explain that mutations in miRNA transcripts represent rare events. However, a combination of loss of heterozygosity (LOH) and mutation was reported as an inactivating event in two cases of CLL. Many miRNAs are frequently located at fragile sites on chromosomes (Calin *et al*., 2004), as well as in minimal regions of LOH, minimal regions of amplification, or common breakpoint regions. Loss of heterozygosity in 13q14.3, intron 4 of *DLEU2*, and downregulation of miR-16-1-15a cluster were observed in the majority of B-CLLs. Amplification of 13q31.3, intron 3 C13orf25, and overexpression of miR-17-92 cluster have been described in follicular lymphoma. Finally, in neuroblastoma and breast cancer the overexpression of miR-21 could be associated with amplification of the region 7q23.2. Therefore, although it seems clear that altered miRNA expression is a characteristic of tumoral cells, it remains to be seen whether miRNA expression changes are a cause or consequence of tumor development.

IDENTIFICATION OF miRNA TARGETS: BIOINFORMATICS AND FUNCTIONAL TOOLS

The knowledge of the gene regulation network by miRNA is just beginning. The effects that miRNA exerts on its targets result in either the repression of mRNA translation of the mRNAs that carry miRNA binding sites in their 3′-UTR regions or mRNA degradation. This means that elucidation of gene regulation derived from miRNA will require application of both transcriptomics and proteomics approaches.

Elaborate single-nucleotide mutation studies of several known miRNAs have been used to investigate the binding pattern of these miRNAs to their target. A clear conclusion from these studies is the importance of the 5′-end segment of the miRNAs, referred to as its seed. This seed, with a length of 6–8 nucleotides, has been shown to be critical, and in some cases sufficient, to suppress the miRNA targets. Its 5′-end is typically unpaired or starts with a uracil and preferably does not contain G:U wobbles. However, recent evidence indicates that the 3′-end of a miRNA may compensate for insufficient base-pairing of its 5′ seed, and is thus named the 3′-compensatory site. In addition, mutation studies have been used to explore the role of multiple binding sites of miRNAs to the same mRNA target, and such studies show that their function may depend on binding to these multiple binding sites. The identification of regulation sites

for different miRNAs in the same 3′-UTR could indicate that an mRNA is regulated independently by these miRNAs in different tissues or different cellular statuses. Moreover, recent studies suggest that the bidimensional structure of miRNA binding sites in the mRNA target and their neighboring regions must be sufficiently unstable to be accessible to miRNA binding.

Several independent groups have established computational algorithms designed to predict target genes of miRNA sequences. Stark *et al*. (2003) used a target prediction algorithm to detect Drosophila miRNA targets based on detecting complementary sequences of the 5′-end 8-nucleotide seed of the miRNA (http://www.miRNA.embl.de). These sequences are evolutionarily conserved across species, and MFold was used to calculate the thermodynamic stability of the binding. Lewis *et al*. (2003) used an algorithm called TargetScan to identify mammalian miRNA targets (http://www.targetscan.org/). This algorithm seeks a strong seven-nucleotide seed, starting from the second nucleotide from the 5′-end, uses RNAFold to calculate the thermodynamic free-energy of the binding, and scores both a single binding site and multiple binding sites. TargetScan requires a shorter seed that is preceded by adenosine and is located in a short region of conservation. The algorithm specifically recovers all known miRNA targets and is estimated to have a 22–31% false-positive rate. Another algorithm, called miRANDA, identifies miRNA targets in Drosophila (Enright *et al*., 2003) and humans (John *et al*., 2004) (http://www.microrna.org/). The algorithm uses a position-weighted matrix to emphasize binding of the miRNA 5′-end segment more than its 3′-end segment, uses RNAFold for free-energy calculation, and relies on evolutionary conservation of the binding sites. It is estimated to have a 24–39% false-positive rate. Pictar is an algorithm designed by Krek *et al*. (2005) and Grun *et al*. (2005) to identify miRNA targets in vertebrates, *C. elegans*, and Drosophila (http://pictar.bio.nyu.edu/). This algorithm is designed to identify multiple binding sites targeted by a single miRNA, and those coregulated by several miRNAs in a coordinated manner. This algorithm is estimated to have 30% false-positive rate.

At present, it is difficult to judge which of the algorithms produce a more reliable target prediction. Almost all algorithms use evolutionary conservation of target sites as a filter; biologically important targets can be discarded if true targets are not conserved in the considered species. Prediction of miRNA targets could become even more extensive as recent experimental evidence suggests that binding sites of miRNAs to 3′UTRs do not necessarily have to be conserved among different species (Miranda *et al*., 2006). Binding of multiple miRNAs to one target could further increase the complexity of target predictions. Moreover, it could also be true that sites for different miRNAs in the same target indicate that the mRNA is regulated independently by these miRNAs in different tissues or during development. Therefore, it seems important that systematic experiments are carried out that test targets predicted by algorithms. TarBase offers a comprehensive set of experimentally supported targets in eight different species (Sethupathy *et al*., 2006). For every target site that has gained experimental support, TarBase describes the miRNA that binds it, the kind of inhibition that miRNA induces, its single-site sufficiency status, its genomic location, the types of experiments that were conducted to

support it, and the manuscript from which all of these data were extracted (http://www.diana.pcbi.upenn.edu/tarbase.html).

To date, several methods have been established to show miRNA regulating their putative targets. Most tests use luciferase reporter constructs containing target 3′-UTRs with the putative binding site downstream of the reporter coding region (Felli *et al*., 2005). These constructs are used to transfect cells expressing the relevant miRNA, along with vectors carrying mutant versions of binding sites. Evidence for miRNA activity can be established when wild-type reporters have less activity than their respective mutants. A complementary approach is to use studies in which the miRNA can be inhibited using antisense 2′-O-methyl modified oligoribonucleotides that are complementary to the targeted miRNA (Poy *et al*., 2004). Increased luciferase activity in reporter assays or upregulated gene expression of the endogenous target protein indicates inhibition of miRNA activity. Induced expression of miRNA can be achieved by transfection of double-stranded RNA molecules that mimic the Dicer cleavage product. Several groups have introduced miRNA-expression plasmids into adenovirus or retrovirus systems to overcome the low efficiency in primary cells or to deliver miRNA to mouse tissues *in vivo*. An additional approach to study the role of individual miRNAs is to restore the expression of specific miRNAs in a Dicer-null system.

TECHNOLOGY TO QUANTIFY miRNAS

Until very recently, the most common method for quantifying miRNA was Northern blotting. The disadvantages are its low throughput and limited sensitivity for detecting rare miRNAs, and a large amount of RNA is required. However, Northern blotting is still regarded as the gold standard for miRNA validation and confirmation of high-throughput data. The sensitivity of detection of miRNAs by Northern blot has been increased by tenfold using locked nucleic acid (LNA)-modified oligonucleotide probes. LNA probes exhibit higher thermal stability and show improved hybridization properties against complementary RNA targets. Fortunately, in the past 2 years there has been significant progress in performance and fine-tuning of several validation approaches, resulting in higher sensitivity and higher throughput capabilities.

At present, other hybridization techniques are available for miRNA quantification, including dot blotting, RNase protection assay, primer extension analysis, and Invader assay. Large-scale cDNA cloning can also provide information on the relative expression levels of miRNAs in diverse samples. However, most of these techniques involve laborious procedures, making it difficult to determine the level of all known miRNAs. Moreover, with these technologies, less abundant miRNAs could routinely escape detection.

Recent development of easy quantification methods has enabled large-scale expression profiling of miRNAs. Currently, the most widely used method is based on microarrays. Although microarray is a powerful method for high throughput analysis, the small size of miRNAs poses a challenge for conventional microarray techniques because it is difficult to create a single hybridization condition suitable for all miRNAs on the chip. Thus, some of the microarrays employ probes that

are complementary to pre-miRNAs rather than mature miRNAs. However, because maturation of miRNA is often regulated, the level of pre-miRNA does not always correlate with that of mature miRNA. Recently developed microarrays detect mature miRNA by employing antisense oligonucleotides that bind specifically to the mature miRNA sequence. However, the problem of potential cross-hybridization of related miRNAs remains unresolved. Moreover, it should be mentioned that microarrays in most cases are not as quantitative as Northern blotting, so it is difficult to determine precisely the relative abundance of miRNAs. Therefore, the problem of a narrow dynamic range remains to be overcome. Microarrays, Invader assays, and bead-based miRNA expression do not amplify miRNA, and thus the sensitivity is often compromised. Moreover, for microarray studies, the short nature of mature miRNAs raises concerns regarding probe specificity. This can be improved by performing hybridization in solution.

Real-Time PCR is the gold standard for gene expression quantification. miRNA quantification by this technology is unquestionably the most sensitive method. However, it could be an important disadvantage to use real-time PCR in high-throughput analysis if the number of miRNAs increases as expected. In this case, real-time PCR will be less practical than microarrays. The technical difficulty also stems from the short length of miRNA (22 nucleotides). Initial methods to detect miRNA by real-time PCR were based on the detection of miRNA precursors rather than the mature miRNAs. It was necessary to modify the method to specifically detect mature miRNA without introducing experimental bias.

Actually, a novel miRNA quantification method has been developed using retrotranscription (RT) primers containing a partial stem-loop structure, followed by TaqMan PCR analysis. Stem-loop RT primers are better than conventional ones in terms of RT efficiency and specificity. TaqMan miRNA assays are specific for mature miRNAs and discriminate between related miRNAs that differ by as little as one nucleotide. Furthermore, they are not affected by genomic DNA contamination. Precise quantification is achieved routinely with as little as 25 pg of total RNA for most miRNAs. In fact, the high sensitivity, specificity, and precision of this method allows for direct analysis of a single cell without nucleic acid purification. Like standard TaqMan gene expression assays, TaqMan miRNA assays exhibit a dynamic range of seven orders of magnitude.

The most recent innovation in miRNA detection involves the bead-based flow cytometric method. Each individual bead is marked with fluorescent tags and coupled to probes that are miRNAs of interest. miRNAs are ligated to 5′ and 3′ adaptors, reverse-transcribed, amplified by PCR using a common biotinylated primer, hybridized to the capture beads, and stained with streptavidin–phycoerythrin. The beads are then analyzed using a flow cytometer capable of measuring bead color (representing miRNA identity) and phycoerythrin intensity (representing miRNA abundance). Because hybridization takes place in solution, this method offers more specific detection of closely related miRNAs compared with conventional glass-slide microarrays. The complicated procedure, however, needs to be improved. Importantly, a study performed by Lu *et al.* (2005) analyzed 217 known human miRNAs in 218 samples

from normal and tumor tissues, demonstrating a surprisingly accurate correlation of miRNAs with the development and differentiation of tumors.

Another new procedure called the RNA-primed array-based Klenow enzyme (RAKE) assay has been developed (Nelson *et al*., 2004). The DNA oligonucleotide probe on the slide contains the sequences antisense to miRNA and the universal spacer sequences. When miRNA binds to the probe, miRNA serves as a primer for extension upon the addition of the Klenow enzyme, and generates a double-stranded fragment with incorporated tagged nucleotides, which is easily detected. This method is particularly useful when closely related miRNAs need to be analyzed separately, because miRNAs with mismatches at the 3′ end cannot be extended.

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED miRNA IN COLORECTAL CANCER USING REAL-TIME PCR

Experimental Workflow for miRNA Expression Analysis by Real-Time PCR

The first step in the analysis of miRNAs is purification of the RNA from a biological sample. Most RNA isolation kits were developed to recover messenger RNA, while disregarding smaller molecules such as miRNAs. Isolation of miRNA begins when total RNA that includes the small RNA fraction is isolated from the samples of interest. However, not all isolation methods retain the small RNA fraction. The standard glass fiber filter (GFF) or silicate adsorption method employed by most RNA isolation kits is inefficient at recovering small RNAs. Therefore, it is important to use RNA isolation methods specifically adapted to retain small RNAs.

The short length of mature miRNAs (~ 22 nt) prohibits conventional design of a random-primed RT step followed by a specific real-time assay. The stem-loop primer improves the specificity for only mature miRNA targets, and formation of a RT primer-mature miRNA chimera, extending the 5′-end of the miRNA. The resulting longer RT amplicon presents a template amenable to standard real-time PCR, using TaqMan assays. The real-time PCR reaction involves a forward primer, a reverse primer, and a TaqMan probe, which quantify the number of mature miRNA molecules present in a sample based on fluorescent emission of a reporter dye. The miRNA assays are able to distinguish between the hairpin structure of precursor miRNA and the short mature miRNA molecules. A stem-loop structure, engineered into the reverse transcription primer and specific to the 3′-end of the mature miRNA, presumably creates steric hindrance to prevent priming of the precursor miRNA. As a result, the assays detect and quantify only mature miRNA molecules, the form capable of interacting with target mRNA molecules.

miRNA Expression in CRC Cell Lines

In order to investigate differential expression of miRNA in human colorectal cancer, we analyzed the expression of 156 mature miRNAs in total RNA extracted from 15 CRC cell lines by real-time PCR using TaqMan MicroRNA Assay kit (Applied

Biosystems) (Bandres *et al*., 2006). We compared their miRNA expression profiles with those of CCD-18Co (normal human colon cell line).

It is generally accepted that gene expression levels should be normalized by a carefully selected stable internal control gene. However, to validate the presumed stable expression of a given control gene, prior knowledge of a reliable measure to normalize this gene in order to remove any nonspecific variation is required. To address this problem, we assessed the normalization data using three different approaches: let-7a (a miRNA that the manufacturer suggested may be useful as an endogenous miRNA control according to their preliminary data across several human tissues and cell lines), 18s rRNA (the most stable housekeeping gene in our CRC samples), and global median-normalization similar to microarray analysis. After normalization, data were expressed as $\log_{10}$ relative quantity (RQ) of target miRNA relative to control sample. The different normalization approaches reveal similar results.

Analysis of k-means clustering to identify a group of 22 and 22 miRNAs homogeneously upregulated and downregulated, respectively, in all colorectal cancer cell lines, and commonly detected with the three different normalization approaches used (Table 6.1). Remarkably, this classification included only those miRNA whose expressions were most prominently altered, and in addition the expression of this group of miRNAs was highly reproducible in all cell lines analyzed. Interestingly, clustering analysis divided CRC cell lines in two different groups. Analysis of different common genetic alterations described in colorectal cancer, including activation of oncogenes (*KRAS*, *BRAF*), inactivation of tumor suppressor genes (*TP53*), and microsatellite instability status (MSI), showed that these groups could be differentiated according to the presence of mutations in *KRAS* and *BRAF* genes. One group included DLD1, SW1116, SW620, SW480, HCT116, Lovo, Colo320, LS174, LS513, and LS411 CRC cell lines. All of them, except for LS411 and Colo320, harbored mutations in the *KRAS* gene. On the other hand, the other group includes mainly CRC cell lines with *BRAF* mutations (WiDR, SW1417, Caco2 and RKO). SAM (Significance Analysis of Microarrays) analysis between both groups identified six differentially expressed miRNA. Colorectal cancer cell lines with *KRAS* mutations showed an overexpression of miR-9, miR-9*, miR-95, miR-148a, miR-190, and miR-372, relative to the normal human colon cell line. This overexpression was lower in the colorectal cancer cell lines with mutations in *BRAF*.

As shown in Table 6.1, the fold-change observed in CRC cell lines relative to CDC18Co differed between -4.5 to -1.5 $\log_{10}$ for downregulation and 1.4 and 3.8 $\log_{10}$ for upregulation. Some of the genes encoding miRNA that are modulated in CRC cell lines are located in determined chromosome segments, suggesting that their tumor-specific expression could be due to DNA abnormalities. In this context, we observed a preferential downregulation in region 14q32.31, including miRNA miR-127, miR-370, miR-299, miR-154, miR-154*, miR-323, miR-134, miR-368, and miR-337. By using a computer-assisted approach, Seitz *et al*. (2004) identified 46 potential miRNAs located in human 14q32 domain, 40 of which are organized as a large cluster. Although some of these clustered miRNA genes appear to be encoded by a single-copy DNA sequence, most

Table 6.1. miRNA differentially expressed in CRC cell lines.

	Mean fold-change ($\log_{10}$ RQ)	Chromosome localization	Putative targets associated with colorectal carcinogenesis
hsa-miR-147	−4.56	9q32.3	
hsa-miR-127	−4.27	14q32.31	
hsa-miR-145	−4.13	5q32	TGFRII, APC
hsa-miR-370	−4.05	14q32.31	BAX, AKT1
hsa-miR-299	−3.90	14q32.31	B-CATENIN, CDKN1A
hsa-miR-199a	−3.80	1q24.3	
hsa-miR-154*	−3.71	14q32.31	MLH1
hsa-miR-199-s	−3.64	19p13.2	
hsa-miR-323	−3.56	14q32.31	MSH2
hsa-miR-154	−3.55	14q32.31	
hsa-miR-134	−3.34	14q32.31	
hsa-miR-342	−3.15	14q32.2	
hsa-miR-199a*	−3.06	1q24.3	
hsa-miR-137	−3.05	1p21.3	TGFRII
hsa-miR-368	−3.03	14q32.31	
hsa-miR-130a	−3.02	11q12.1	TGFRII
hsa-miR-214	−2.36	1q24.3	TP53, B-CATENIN, TGFRII, BAX, CDKN2B, EGFR
hsa-miR-337	−2.25	14q32.31	CDKN2A
hsa-miR-125b	−2.20	11q24.1	VEGF, IGFRI, VEGFR
hsa-miR-199b	−2.19	9q34.11	
hsa-miR-133a	−2.11	18q11.2	BAX, K-RAS
hsa-miR-26b	−1.82	2q35	APC
hsa-miR-133b	−1.66		K-RAS
hsa-miR-296	−1.61	20q13.32	
hsa-miR-124b	1.42		MLH1
hsa-miR-338	1.63	17q25.3	
hsa-miR-9*	1.68	5q14.3	TCF4, MSH2
hsa-let-7 g	1.73	3p21.2	TGFRII
hsa-miR-372	1.76	19q13.42	TGFRII, SMAD2, MLH1, AKT1
hsa-miR-182*	1.77		
hsa-miR-219	1.93	6p21.32	TGFRII
hsa-miR-205	2.21	1q32.2	K-RAS, SMAD4, MSH2, PTEN
hsa-miR-194	2.23	1q41	
hsa-miR-142-3p	2.29		APC
hsa-miR-135a	2.36	3p21.2	MSH2
hsa-miR-215	2.42	1q41	IGFRI
hsa-miR-142-5p	2.51	17q23.2	
hsa-miR-135b	2.90	1q32.1	MSH2
hsa-miR-141	3.28	12p13.31	APC, MSH2
hsa-miR-182	3.41	7q32.2	IGFRI
hsa-miR-200b	3.44	1p36.33	MLH1
hsa-miR-200c	3.64	12p13.31	MLH1, SMAD2
hsa-miR-96	3.64	7q32.2	K-RAS
hsa-miR-200a	3.73	1p36.33	MSH2
hsa-miR-203	3.77	14q32.33	

of them are arranged in tandem arrays of closely related sequences.

However, 14q is not a region usually deleted in CRC cancers, although its loss has been associated with disease progression and a worse prognosis. In contrast, we hypothesize that differential expression could be regulated by modulation of their transcription. We think that this hypothesis may be supported by the observation that different "isoforms" of some downregulated and upregulated miRNA in CRC cell lines are located on different chromosomes, and their coordinated expression might reflect the existence of a common target. The expression of mir-200a, mir-200b, and mir-200c, located on two different chromosomes (1 and 12) and with high sequence homology, are upregulated in all CRC cell lines. The analysis of their putative targets showed *MLH1* and *MSH2* as two candidate genes whose transcription could be downregulated by miRNA. Our findings indicate that miRNA expression patterns are closely related to characteristics of tumor-derived cell lines. These patterns may either mark specific biologic characteristics or may mediate specific biologic activities important for the pathobiology of malignant tumors.

miRNA Expression in Colorectal Tumors and Adjacent Non-tumor Tissues

In order to investigate whether miRNAs are differentially expressed in CRC versus normal colon tissues, we analyzed miRNA expression in 12 matched-pairs of tumoral and nontumoral tissues. After testing two different approaches to normalize the cycle threshold (Ct) raw data in CRC cell lines, median-normalization was selected as the method for clinical samples because normal distribution was not required. Meanwhile, in our study in CRC cell lines, no differences were found in let-7a expression between tumoral and normal cell lines; recent evidence identified the let7-family as differentially expressed in CRC and lung cancer. Moreover, global median normalization could provide results more easily comparable with those already published with microarray technology.

To identify miRNA with significant differential expression among CRC samples, two multivariate permutation tests provided in BRB-ArrayTools were performed: Class Comparison between Groups of Arrays and SAM. Fifty-nine miRNAs were significant when the Class Comparison test was applied, 68 miRNAs were significant using the SAM test, and 53 miRNAs were common in both tests. As expected, fold-change observed in clinical samples was less homogeneously distributed among samples that had been already obtained in CRC cell lines. It is not surprising that patient samples are composed of mixed populations, whereas cell lines are clearly more uniform.

We detected an overexpression of miR-19a, miR-21, miR-29a, miR-92, miR-148a, miR-200b, and a downregulation of miR-30c, miR-133a, and miR-145. Expression of some of these miRNAs has been previously reported in B-cell lymphomas, glioblastoma, and lung or breast cancer. Among them, miR-19a and miR-20 are included in the cluster miR-17-92, which is located at intron 3 of C13orf25. The transfection of C13orf25 in lung cancer cell lines enhanced cell growth, and the introduction of miR-17-92 into hema-topoietic stem cells in *Eu-myc* transgenic mice accelerated the formation of lymphoid malignancies. Furthermore, miR-21 has

been described as an antiapoptotic factor in human glioblastoma cell lines. In contrast, other authors report that miR-21 suppression increased growth in HeLa cells without affecting their apoptosis. The different biologic effects of any particular miRNA in different cells could be dependent on the cell-specific repertoire in target genes. Some differentially expressed miRNA in CRC samples have been associated with clinical parameters in other cancers. In particular, miR-145 is progressively downregulated from normal breast to cancer with high proliferation index, and miR-21 is progressively upregulated with high-grade tumor stage.

To identify the smallest set of predictive miRNAs differentiating normal versus cancer tissues, we have used support vector machine (SVM) techniques. We attempted to use the class prediction tool (BRB-Array tools) that creates a multivariate predictor for determining to which of two classes a given sample belongs. Several multivariate classification methods are available, including the Compound Covariate Predictor, Diagonal Linear Discriminate Analysis, Nearest Neighbor Predictor, Nearest Centroid Predictor, and Support Vector Machine Predictor. The classifier is composed for 18 miRNA, 10 downregulated and 8 upregulated, all of them significantly different by Class Comparison and SAM tests.

When we compared expression of these miRNAs in CRC cell lines, 5 of 18 miRNAs were revealed in the k-means analysis as those with the highest fold-changes (in relation to CDC18Co). However, Class Comparison analysis between 15 CRC cell lines and the 12 nontumoral colon tissues identified 13 miRNA altered in both systems: CRC patient samples and CRC cell lines. These results indicate that the miRNA profile in CRC cell lines cannot be used to infer miRNA expression in clinical samples, but the cell lines can be used as a model to validate and perform functional assays of data obtained in clinical samples.

The expression of 5 of 13 miRNAs has already been described that they are altered in CRC, lung and breast cancers, glioblastoma, B-cell lymphoma, and CLL. Among the differentially expressed miRNAs, miR-31, miR-96, miR-133b, miR-135b, miR-145, and miR-183 are the most consistently deregulated in CRC. Two of them, miR-133b and miR-145, were downregulated, and the remaining 4: miR-31, miR-96, miR-135b, and miR-183 were upregulated, suggesting that they may potentially act as tumor suppressor genes or oncogenes, respectively.

miR-145 was identified as a specific miRNA downregulated in colorectal neoplasia, and analysis of their pre-miRNA indicated that this reduction was due to a posttranscriptional process. Recently, Cummins *et al*. (2006) obtained similar results in CRC, and downregulation of miR-145 has also been reported in lung and breast cancers. In our study, expression of miR-145 was not detected in any of 15 CRC cell lines tested, and downregulation was detected in all tumor samples. Another important downregulated miRNA in our study was miR-133b. To our knowledge, this miRNA has not been previously identified as deregulated in cancer. For both downregulated miRNAs (miR-145 and miR-133b), it may be expected that potential targets could include oncogenes or genes encoding proteins with potential oncogenic functions. Indeed, among putative targets for miR-145 with potential

oncogenic functions, Iorio *et al*. (2005) described *MYCN, FOS, YES*, and *FLI*, cell cycle promoters such as cyclins D2 and L1, and MAPK transduction proteins such as *MAP3K3* and *MAPK4K4*. Among putative targets of *miR-133b*, the most notable oncogenic target is *KRAS*. Interestingly, the protooncogene *YES1* and the transduction protein *MAP3K3* were potential targets of both miR-145 and miR-133b.

For the upregulated miRNAs miR-135b, miR-31, miR-96, and miR-183, it may be expected that gene targets belong to the class of tumor suppressor genes. miR-96, miR-182, and miR-183 are located in the same chromosomal region, 7q32.2. miR-182 was not detected as preferentially over-expressed with the most restricted analysis, but its upregulation was clearly observed in CRC cell line analysis (Table 6.1). CHES1 protein was identified as a potential target of both miR-96 and miR-182. Other members of this family, including *FOXF2*, *FOXK2*, *FOXO1A*, *FOXO3A*, and *FOXQ*1, were also found as putative targets of miR-182, miR-183, and miR-96.

Finally, our analysis of a small number of CRC samples compared miRNA expression in tumors according to pathological stage (stage II versus stage IV). The upregulation of miR-31 was significantly higher in stage IV than in stage II CRC samples ($p = 0.028$). The expression levels of miR-31 were higher in the tumor samples and CRC cell lines in comparison with the nontumoral samples and was related to pathological stage, suggesting that this miRNA could contribute to both the tumorogenesis and the acquisition of a more aggressive phenotype in CRC. Other members of the forkhead family of transcription factors, such as *FOXC2* and *FOXP3*, were identified as putative targets of miR-31. Future studies will determine the correlation between these miRNAs and their host genes in CRC. In summary, our results by real-time PCR identified alterations of miRNA expression in CRC, which may deregulate cancer-related genes and would provide potential mechanisms that underlie the carcinogenesis and further acquisition of a more aggressive phenotype in colon cancer.

QUANTIFICATION OF miRNA IN CLINICAL SAMPLES

Given the rapid progress during the past several years in miRNA expression profile detection, it is likely that miRNAs have a promising future in cancer diagnostics.

The utility of miRNA profiling in CLL, lung cancer, thyroid cancer, breast cancer, colorectal cancer, and B-cell lymphoma is now apparent. In CLL and lung cancer, specific expression signatures are associated with either favorable or poor prognosis. Patients falling in the category of unfavourable outcomes may be placed into appropriate clinical trials, treated more aggressively, or receive palliative care depending on the particular case. Likewise, patients with favorable prognoses can benefit by having a better indication of their outcome and avoid potentially harmful treatments. An exciting future prospect is that the miRNA patterns associated with particular outcomes may ultimately provide insights into the underlying etiologies of disease and reveal therapeutic targets. An especially interesting viewpoint is the potential for miRNAs to serve as early markers for cancer initiation or progression. It remains an exciting possibility that neoplastic lesions may

have dysregulation of miRNA levels that could serve as sentinels of tumor initiation. Unfortunately, the reproducible detection of small quantities of such RNA species in the blood or other easily accessible body fluids may prove challenging and will likely require further technical advances. Fortunately, miRNAs, unlike larger RNAs, remain largely intact in routinely collected formalin-fixed, paraffin-embedded clinical samples, enhancing their potential utility and suggesting that their overall levels may be less likely to be affected by storage or collection procedures.

It is important to determine if dysregulated miRNAs control pathways that are essential for tumor growth, because miRNA-regulated proteins might be useful therapeutic targets. At least two examples already suggest that miRNA dysregulation may affect major oncogenes such as c-myc and RAS. Additionally, the recent successes of inhibition of miRNAs at the cellular level suggest the possibility of direct targeting of miRNAs that are amplified or upregulated in patient tumors. Most of these clinical applications will depend on accurate assessment of miRNA profiles in human samples. Examination of individual miRNA scans should be performed by Northern hybridization and specialized real-time PCR, and can be assessed in cellular contexts through *in situ* hybridization. At present, standardization of methodology applied in order to obtain reproducible results between different platforms is necessary for application of miRNA profile in clinical management. A set of commercially available standard miRNAs or samples would be helpful in comparing results among analyses of miRNA profiles for both research and clinical use. Real-time PCR and bead-based flow cytometry may help establish an automatable, high-speed process for miRNA profiling in the near future.

Although still in their infancy, miRNA analyses offer possibilities in tumor classification, disease prognosis, early cancer detection, and therapeutic decision-making. Although clinically relevant miRNA studies are moving forward, it should be renoted that this field is relatively young and many questions remain. The total number of human miRNAs has yet to be determined, the targets of miRNAs and their roles in cellular pathways are unexplored, and the function of dysregulated miRNAs in human cancer remains largely a mystery. That the important functions these small RNAs play in normal biology, is certain that they will have a similarly large role in human cancer.

FUTURE APPLICATIONS AND POTENTIAL LIMITATIONS

The analysis of miRNA expression profiles in cancer cells has revealed that deregulation of these molecules is frequent in a wide variety of tumors. The confirmation of the critical targets responsible for the phenotypic effects of miRNA loss- or gain-of-function will provide potential targets to reduce tumor growth.

Although miRNAs are becoming increasingly recognized as regulatory molecules in human cancers, their involvement in functional responses to environmental changes, such as exposure to chemotherapy, is unknown. Only one article analyzes the changes of miRNA expression observed in response to gemcitabine in cancer cell lines. Meng *et al*. (2006) found that miR-21 and

miR-200b inhibition increased the sensitivity of cholangiocarcinoma cell lines to this drug. The authors found that miR-21 modulated gemcitabine-induced apoptosis by regulation of PTEN expression and PI3-kinase activation. These results suggest that miRNA could be included as pharmacogenomic markers. However, analysis of miRNA expression in clinical samples and their association with response to treatment has been not assessed.

If miRNAs represent cancer players, they should be considered as potential therapeutic targets. It might be possible to regulate miRNA expression or inject miRNAs to regulate cancer formation, similar to the use of antisense mRNAs and RNAi, which are widely used as tools for studying gene functions and, in some cases, gene therapy. miRNA therapeutics should borrow techniques from the antisense research community, which has been developing therapeutic RNAs for more than a decade. Recent work has demonstrated that modified antisense RNAs can inhibit miRNA function in adult mouse. The modulation of aberrantly expressed miRNA may be useful to improve responses to cytotoxic therapies

To date, most studies of the genetic mutations that contribute to tumor formation have focused on alterations in the sequence, gene structure, copy number, and expression of protein-coding genes. However, the genome generates a diversity of non-coding RNAs, many of which have unknown functions. MicroRNAs act through the RNAi pathway to regulate the expression of protein-coding genes, which they recognize through complementary base pairing. Accumulating data suggest causal roles for microRNAs in human cancer, including observations of microRNA genes at tumor-associated chromosomal lesions and direct demonstrations that altered expression of microRNAs can accelerate tumor development. However, as new targeted therapies are currently more focused on a wider spectrum of action, future therapeutic approaches regarding miRNAs must consider the use of clusters acting on the control of different crucial proteins involved in the aggressive behavior of cancer. Indeed, promising strategies are expected in the use of miRNAs in monotherapy or in combination with other treatment options.

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B. Treatment

7
Colorectal Cancer: Optimization of the Combination of 5-Fluorouracil and Irinotecan

Carlo Barone, Matteo Landriscina, and Alessandra Cassano

INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of cancer in both men and women worldwide (Goldberg, 2005). Upon diagnosis, 19% of CRC cases are metastatic, and while the overall 5-year survival rate for patients with early CRC is ~ 60%, the rate drops to 10% or less in patients with advanced disease (Goldberg, 2005).

5-Fluorouracil (FU) has been for many years the backbone of therapy for CRC since it demonstrated to improve patients overall survival either in the adjuvant setting or in the metastatic disease (Goldberg, 2005). Based on the evidence that FU has a schedule-dependent activity, various strategies have been evaluated to improve their cytotoxic effects, such as changing the dose, the schedule and route of administration or combining with the biomodulator leucovorin, overall obtaining response rates of ~ 15–25% (Venook, 2005).

In the last 10 years newer chemotherapeutic agents, such as irinotecan (IRI) and oxaliplatin (l-OHP), used as monotherapy or in combination with FU, have had a significant impact on treatment strategies for patients with CRC and, noteworthy, these new regimens have improved patients outcome either in the adjuvant setting or in the advanced disease (Venook, 2005). Indeed, the FOCUS trial, which enrolled 2,135 patients with advanced CRC, demonstrated the superiority of doublet regimens (i.e., combination of FU with either IRI or OHP) used as first or second-line therapy over sequential singlet therapy (Seymour MT *et al*., 2007). Furthermore, it is very well established that regimens combining FU with IRI or l-OHP are equally effective in terms of response rate and overall survival (Tournigand *et al*., 2004) and represent the standard first line treatment in advanced CRC (Goldberg, 2005). However, the analysis of seven phase III trials in advanced CRC patients suggested that the exposure to all three drugs, regardless of their sequence, is a key element able to extend the overall survival of patients to 18–21 months (Grothey *et al*., 2004). Finally, a recent large multicenter trial demonstrated that the FOLFOX-4 regimen (l-OHP combined with the bimonthly regimen of FU and leucovorin) is superior to bimonthly FU and leucovorin as adjuvant therapy for CRC (André *et al*., 2004) and is, at present, widely accepted as a new standard therapy in III stage CRC.

However, even though several significant progresses have been achieved, some issues are still open in the treatment of CRC, and several new studies have been performed or are still ongoing with the aim to find the best combination and the best sequence of administration of these chemotherapeutic agents. Indeed, the results that are emerging from these studies reinforce the need of optimizing doses and schedules of doublet chemotherapy in order to deliver the three drugs sequentially and obtain maximal cytotoxic activity against tumor cells and minimal toxicity to the normal cells.

In the last few years, the scenario has been further complicated by the advent of new molecular-targeted agents (Venook, 2005). Indeed, the monoclonal antibody anti-VEGF, bevacizumab, and the monoclonal antibody anti-EGFR-1, cetuximab, became available in the clinical management of advanced CRC. Bevacizumab has been proved to be clinically active in combination with IRI/FU or l-OHP/FU, respectively, as a first- or a second-line therapy in metastatic CRC (Hurwitz *et al*., 2004; Giantonio *et al*., 2007), whereas the combination of IRI and cetuximab is a salvage chemotherapy in IRI-resistant patients (Cunningham *et al*., 2004). Because these novel molecular-targeted agents are characterized by a cytostatic mechanism of action and have been demonstrated to synergize with traditional cytotoxic drugs (Prewett *et al*., 2002), the issue to optimize the combination of cytotoxic and molecular-targeted drugs is becoming more critical to maximize their efficacy in the management of CRC patients. This chapter will focus on the issues related to the combination of IRI and FU and will review the more significant studies aimed to improve its antitumor activity either at preclinical or at clinical level.

THE COMBINATION OF 5-FLUOROURACIL AND IRINOTECAN IN HUMAN COLORECTAL CANCER

Several combination regimens with FU and IRI have been evaluated as first line therapy of advanced CRC, achieving a response rate of 30–50% and an overall survival of 14–20 months (Venook, 2005). While most of the regimens combining l-OHP and FU differ only marginally, the combinations of IRI and FU are characterized by major differences in terms of doses and schedules (Venook, 2005; Saltz *et al*., 2000; Douillard *et al*., 2000). Interestingly, three regimens of IRI and FU (IFL, FOLFIRI and AIO + IRI) have been evaluated in phase III studies (Saltz *et al*., 2000; Douillard *et al*., 2000; Köhne *et al*., 2005), whereas several other different schedules have been proposed (Venook, 2005). These phase III clinical trials clearly demonstrated the superiority of IRI combined with FU/leucovorin compared to regimens consisting of FU/leucovorin alone (Fuchs *et al*., 2006). Indeed, two pivotal phase III multicenter trials indicated that the addition of IRI to FU/leucovorin administered either as bolus or as a continuous infusion resulted in a doubling of the tumor response rate, in a prolonged median survival for patients receiving the combination and a relative risk of death substantially reduced compared to FU/leucovorin alone (Saltz *et al*., 2000; Douillard *et al*., 2000). Interestingly, in the study of Saltz *et al*. (2000), IRI was

combined with bolus FU/leucovorin in a weekly schedule (IFL), whereas in the Douillard schedule, IRI was combined with infusional FU/leucovorin (FOLFIRI). It is noteworthy that the regimens with IRI and infusional FU were associated with reduced toxicity compared to the IFL regimen (Fuchs *et al*., 2006).

These data have been confirmed by a third phase III trial, conducted by Köhne *et al*. (2005), which evaluated the combination of IRI with the AIO infusional FU regimen in chemonaive metastatic CRC patients. This study demonstrated a significant improvement in progression-free and overall survival with the combination therapy and confirmed the low toxicity profile of schedules with infusional FU. Thus, based on these evidences the combination of IRI and FU/leucovorin can be considered one option for first-line treatment in metastatic CRC. However, because there are no randomized clinical trials which compared these different FU/IRI schedules, we do not know whether one regimen is better that the others.

THE CLINICAL PHARMACOLOGY OF THE INTERACTION BETWEEN 5-FLUOROURACIL AND IRINOTECAN

Several studies evaluated at preclinical level the pharmacological interaction between IRI and FU (Guichard *et al*., 1998; Mans *et al*., 1999; Barone *et al*., 2007), and, indeed, all of them suggest that the sequential administration of IRI before FU produces additive or synergistic effects in all colon carcinoma cell lines tested (Guichard *et al*., 1998; Mans *et al*., 1999), whereas the sequential exposure of cells to FU before IRI or the simultaneous two-drug treatment produces antagonistic or only additive activity, depending on the colon tumor cell model (Mans *et al*., 1999). Similar evidence has also been obtained *in vivo*, in athymic mice xenografts of colon carcinoma cells (Guichard *et al*., 1998), where higher antitumoral activity was noted when the two drugs were administered sequentially, in comparison with simultaneous administration.

Guichard *et al*. (1998) demonstrated that the preincubation of colon carcinoma cells with IRI before FU enhances the incorporation of FU derivatives into the DNA and the formation of DNA-protein complexes with a parallel more persistent decrease in thymidylate synthase (TS) activity and a more lasting S-phase arrest. Furthermore, diminished DNA synthesis, elevated dTTP pools, inhibition of dUMP synthesis and increased DNA damages were also observed in colon carcinoma cells when cells were preexposed to IRI before FU (Mans *et al*., 1999).

A similar pharmacological interaction has been described by Aschele *et al*. (1998) by studying the cytotoxic profile of the combination of another TS-inhibitory agent, raltitrexed, and IRI in human colon carcinoma cell lines. These authors observed that the sequential short-term exposure to SN-38, the active metabolite of IRI, followed by raltitrexed results in synergistic cytotoxicity at broad dose-effect ranges and a 24-h interval between the two agents enhances the magnitude of this synergism. By contrast, the opposite sequence or the simultaneous exposure produces significantly less potentiation (Aschele *et al*., 1998).

Our group has been recently involved in studying the interaction between SN-38 and FU in human colon carcinoma HT-29 cells, a cell lines with non-functional p53, in order to obtain *in vitro* evidence for optimizing chemotherapeutic schedules. We observed that the sequential exposure of colon carcinoma cells to the two agents produces a supra-additive effect with maximal cytotoxic activity when cells are preexposed to SN-38 before FU, whereas the concomitant exposure to both drugs produces only additive effects. Interestingly, this synergism depends on the extent of cell exposure to FU and the interval between the two drugs. Indeed, it is possible to potentiate this synergism of action by prolonging the exposure of tumor cells to FU up to 96 h and by administering sequentially the two agents with minimal interval in between. Furthermore, the sequential exposure of cells to SN-38 before continuous infusion FU elicits the maximal increase in apoptotic cell death (Barone *et al*., 2007). These results are consistent with the evidence reported by te Poele and Joel (1999), who demonstrated that the cytotoxic activity of IRI depends on the schedule and the p53 status of tumor cells. Indeed, these authors observed significantly higher level of apoptosis for prolonged exposure to SN-38 in p53-deficient human colon carcinoma cells, and for shorter exposure to higher concentrations in p53 wild-type colon carcinoma cells.

The sequential short-term exposure to SN-38 followed by prolonged treatment with FU also results in a significantly higher increase in the S phase fraction (Barone *et al*., 2007). Indeed, while FU produces an arrest of cells in S-phase of the cell cycle and SN-38 produces an arrest in the phase G2-M (Yoshikawa *et al*., 2001; Barone *et al*., 2007), tumor cells sequentially exposed to SN-38 followed by FU exhibit a significantly higher increase in the S phase fraction with no arrest in the G2-M phase (Barone *et al*., 2007). These findings suggest that preincubation of colon carcinoma cells with SN-38 facilitates in turn a more prolonged inhibition of TS by FU, an increase in the incorporation of FU derivatives into DNA, an enhanced and persistent S-phase arrest, and apoptotic cell death.

This hypothetical mechanism of action provides a molecular rationale to the observation that the synergistic activity of the SN-38 and FU sequence is partially conserved in colon carcinoma cells resistant to FU and characterized by increased levels of TS (Barone *et al*., 2007). It is also in agreement with the clinical well-known evidence that the FU/IRI-based combination therapy is effective in patients pretreated with FU (André *et al*., 1999; Tsavaris *et al*., 2007), and whose tumors are generally characterized by increased levels of TS (Wong *et al*., 2001). Furthermore, these results suggest that IRI-resistant CRC cells may be more sensitive to schedules with infusional FU, although the molecular mechanism of this synergism is still unclear.

The most commonly utilized schedules with IRI and FU/lecovorin are FOLFIRI and IFL regimens, which are characterized by sequential administration of IRI followed by FU bolus and/or continuous infusion, and both of them have been designed based on the preclinical data described above (Guichard *et al*., 1998; Mans *et al*., 1999). However, considering that IRI half-life is ~ 10 h, both regimens also combine the two drugs simultaneously in a weekly (IFL regimen) or biweekly (FOLFIRI regimen) schedule (Douillard *et al*., 2000; Saltz *et al*., 2000), a condition

that may produce an antagonism of action. Thus, it is reasonable to hypothesize that different schedules which minimize the risk of a negative interaction between the two agents may result in a better clinical activity.

THE CLINICAL OPTIMIZATION OF THE 5-FLUOROURACIL AND IRINOTECAN COMBINATION

Several options have been proposed with the aim to improve the efficacy of standard two-drug regimens for advanced CRC (Venook, 2005). Some studies evaluated the combination of IRI, l-OHP and FU concurrently in a single regimen; the rationale being that nonspecific resistance to therapy may develop after a first line therapy. Indeed, while studies demonstrated that three-drug regimens achieve very high response rates (50–70%), the superiority of the three-drugs regimens in terms of overall survival was confirmed only by one phase III study (Falcone *et al*., 2007). By contrast, patients treated with three-drugs schedules reported significant dose-limiting toxicities such as neutropenia and diarrhoea (Souglakos *et al*., 2002; Goetz *et al*., 2003), arising the question regarding tolerability of such regimens.

Other studies evaluated the combination of FU with l-OHP and/or IRI infused as chronomodulated regimens in which anticancer drugs are administered with optimal timing according to circadian rhythms of anticancer action and those of adverse effects on normal cells. These studies have shown benefit of chronomodulation for single agents 5-FU (Cure *et al*., 2002) and L-OHP (Lévi *et al*., 1993) and for the combination of IRI with chronomodulated OHP and FU, reporting interesting response rates and optimal toxicity profiles (Garufi *et al*., 2003). The role for chronomodulation in CPT-11 administration in combination with chronomodulated FU schedule was evaluated in a randomised phase II trial in advanced colorectal cancer patients. This study compared standard 1-h infusion with a 6-h sinusoidal chronomodulated CPT-11 infusion followed by a 4-day chronomodulated FU and leucovorin (Garufi *et al*., 2006). The trial demonstrated that chronomodulation modality is one of the best possible options to combine CPT-11 with FU and FA because the combination was absolutely safe with regard to haematological toxicity, had an excellent tolerability profile with no neutropenia, febrile neutropenia, hospital admissions, or toxic death (Garufi *et al*., 2006). However, while these regimens exhibited promising results and safe toxicity profile, these studies raised the question regarding the feasibility of chronomodulated chemotherapy in the daily management of CRC patients.

Based on the preclinical evidence described in the previous paragraph and considering the low toxicity profile of infusional FU (Poplin *et al*., 2005), our group evaluated a modified-IRI/FU schedule with IRI administered on day 1 followed by a 4 or 5 days-infusion of FU starting on day 2. This schedule was designed with the aims to: (1) use the sequence that in preclinical experiments demonstrated the highest synergism of action (i.e., SN-30 followed by FU), (2) to avoid any simultaneous exposure of tumor cells to the two agents, and (3) to combine the short term exposure to SN-38 with the prolonged exposure to FU, a condition that elicited the highest rates of apoptosis and increase in S-phase *in vitro* (Barone *et al*., 2007). We tested this alternative FU/IRI-based regimen as first line

treatment in a phase I trial in 25 patients with advanced CRC, evaluating three dose levels of IRI and two of FU in a 3-weekly schedule. Compared to the commonly used two-drug regimens (Venook, 2005), our schedule demonstrated to be feasible and did not increase either the hematological toxicity or the rate of high grade diarrhoea and stomatitis. This is even more relevant considering the toxicity profile of some traditional combination regimens of IRI and FU (i.e., IFL) in which a large proportion of patients experienced grade III–IV hematologic and nonhematologic toxicities (Saltz *et al*., 2000).

The maximum tolerated dose was not reached because two dose-limiting toxicities at the same dose level were not observed. However, at the highest dose level, the theoretical weekly dose intensities of IRI and FU was very similar to the dose intensities of IRI and FU in FOLFIRI and IFL regimens (Saltz *et al*., 2000; Douillard *et al*., 2000), suggesting that these dose levels may deserve to be used in phase II studies. The schedule obtained a response rate > 50%, a disease control rate of 80% and a time to progression of 7 months. These results are promising even though they have been achieved in a dose-escalating phase I trial whose major aim was not the evaluation of the antitumor activity. However, taking into account that in the first dose level we did not observe any response, probably because IRI was underdosed, these findings are even more significant. Indeed, considering only patients enrolled between the second and the fifth dose levels, the overall response and the disease control rates reach 61.9% and 85.7%, respectively (Barone *et al*., 2007).

Similar results have recently been achieved in a phase I dose-escalating trial of IRI and continuous infusion FU as first line treatment of metastatic colorectal cancer. Interestingly, the combination was well tolerated and demonstrated a significant clinical activity, obtaining an overall response rate of 55%, a clinical response benefit of 82%, and a time to progression of 8 months (Saunders *et al*., 2004). Furthermore, Ficorella *et al*. (2006) demonstrated in a phase I dose-finding study that the combination of bimonthly 12-h infusion of CPT-11 followed by 4-days infusion of FU represent a schedule highly tolerated and with a promising clinical activity.

Thus, the results of these studies clearly suggest that the schedule of administration of FU and IRI is critical to achieve the maximal supraadditive cytotoxic activity and that the lack of a full synergism in some traditional schedules of IRI and FU may depend on the use of bolus FU (i.e., IFL) (Saltz *et al*., 2000) and/or the need to optimize the sequence of administration of the two agents (i.e., FOLFIRI) (Douillard *et al*., 2000). Interestingly, it seems possible to improve the clinical activity of the FU/IRI combination by avoiding simultaneous administration of the two drugs and/or by prolonging the infusion of FU.

INTEGRATION OF IRINOTECAN/ 5-FLUOROURACIL REGIMENS WITH CONDENSED AGENTS

Advances in chemotherapeutic agents have led to better outcomes for patients with advanced CRC. However, chemotherapeutic agents are limited by their lack of specificity and are often associated with frequent and potentially severe dose-limiting

toxicities. Therefore, a major goal in clinical oncology research is to develop more effective, better-tolerated treatments that specifically target the processes pivotal to tumorigenesis and metastasis. In the last few years the advances in the understanding of molecular biology have led to the development of agents that target tumor-specific pathways and act by a cytostatic mechanism. Two of these molecules (i.e., the anti-VEGF monoclonal antibody, bevacizumab, and the anti-EGFR-1 monoclonal antibody, cetuximab) are already having a significant impact on treatment strategies for metastatic CRC and are under evaluation in the adjuvant setting (Venook, 2005).

Angiogenesis, the formation of new blood vessels from preexisting vessels, allows tumors to absorb nutrients and oxygen for their further growth and development, and facilitates migration of tumor cells to access the systemic circulation and establish metastases. The switch of a tumor to an angiogenic phenotype is caused by an increased production of proangiogenic factors, including VEGF, basic and acidic fibroblast growth factor, and a decrease in angiogenic inhibitors (Hanahan and Folkman, 1996). VEGF is a specific mitogen for the endothelial cell and acts as a survival factor through the inhibition of apoptosis, and also playing an important role in mobilizing endothelial cell precursors to sites of angiogenesis (Ferrara, 2001). VEGF is upregulated in most human tumors, including colorectal cancer.

Several attempts have been evaluated to target VEGF, although VEGF blockade with monoclonal antibodies is the most studied approach. Bevacizumab is an anti-VEGF humanized monoclonal antibody, and is the most advanced agent of its class in clinical development. Several studies have examined bevacizumab in combination with chemotherapy in the first- and second-line settings in patients with metastatic CRC, reporting extremely interesting results. One phase III trial evaluated the efficacy and the safety of bevacizumab in combination with FU/IRI-based chemotherapy (IFL regimen). The addition of bevacizumab to IFL resulted in a significantly longer survival time, by almost 5 months, in a significantly greater overall response rate, duration of response, and progression-free survival time. Survival benefit has been observed for all patient subgroups, independently of second-line therapy (Hurwitz *et al*., 2004). Other clinical trials are in progress to evaluate the addition of bevacizumab to FOLFIRI or OHP-based schedules. Recently, the addition of bevacizumab to oxaliplatin, fluorouracil, and leucovorin resulted in an improved survival duration in a phase III trial in patients with previously-treated metastatic CRC (Giantonio *et al*., 2007). Surprisingly, however, in untreated patients the addition of bevacizumab to FOLFOX or XELOX (capecitabine/oxaliplatin) has significantly increased progression free but not overall survival (Saltz *et al*., 2007).

The EGFR-1 signaling pathway is thought to play a pivotal role in tumor growth and progression of various human neoplasms, including colorectal cancer. EGFR-1 belongs to the HER family of receptors and can bind several ligands which induce receptor homo- or heterodimerization with another EGFR-1 receptor or other HER family members. Various studies have demonstrated that EGFR-1 signaling is dysregulated in colorectal cancer and other tumor types and that the overexpression of EGFR-1 correlates

with disease progression, poor prognosis, and reduced sensitivity to chemotherapy (Mendelsohn and Baselga, 2000). Therefore, EGFR-1 is considered a molecular target in CRC therapy and several strategies have been developed to target EGFR-1, including small-molecule TK inhibitors (i.e., erlotinib) and monoclonal antibodies anti-EGFR-1 (i.e., cetuximab and panitumumab). Cetuximab exerts its antitumor effects through ligand-independent processes, stimulating receptor internalization and combination therapy with cetuximab and chemotherapeutic agents leads to synergistic antitumor activity (Prewett *et al*., 2002).

Cunningham *et al*. (2004) evaluated cetuximab alone or in combination with IRI-based chemotherapy in patients with irinotecan-refractory metastatic CRC, demonstrating that the inhibition of the EGFR-1 pathway results in the restoration of sensitivity to IRI by reverting mechanisms of chemoresistance. Recently, the combination of cetuximab and IRI/FU/folinic acid was proven to increase response rate and prolong progression-free survival in the first-line treatment of patients with metastatic CRC, reducing the relative risk of progression by ~ 15% (Van Cutsem *et al*., 2007).

These results suggest that the combination of IRI/FU schedules with molecular-targeted agents which block mechanisms critical for tumor progression, such as VEGF or EGFR-1 signaling, results in a strong synergism of action and, therefore, in an improvement of the clinical benefit. Much evidence suggests that this supraadditive effect depends either on the blockage of tumor-specific molecular mechanisms responsible for cancer cell proliferation, or on the inhibition of survival anti-apoptotic mechanisms which trigger the resistance of tumor cells to traditional chemotherapeutic agents (Peng *et al*., 2006). In such perspective, the sequence of the combination of these novel molecular-targeted agents with traditional anticancer drugs may be critical to achieve the best interaction. Thus, new studies are needed to understand whether it is possible to further improve the results already obtained by optimizing these novel combination regimens.

THE PHARMACOGENOMIC OF THE 5-FLUOROURACIL AND IRINOTECAN COMBINATION

The combination regimens commonly used in the management of CRC patients can reach an objective response rate of ~ 40–50% and newer schedules have promised even better results (Venook, 2005). However, these new combinations remain inactive in approximately half of the patients, and, in addition, resistance to treatments appears in almost all the patients who initially were responders. Thus, a major clinical challenge is to identify the subset of patients who could benefit from chemotherapy, both in metastatic or in adjuvant setting.

There have been many attempts to determine predictive factors for response. Several studies have demonstrated that alterations in gene expression, protein expression, and polymorphic variants in genes encoding for thymidylate synthase, dihydropyrimidine dehydrogenase, and thymidine phosphorylase can predict the response to FU (Iacopetta *et al*., 2001; Kornmann *et al*., 2003; Ciaparrone *et al*., 2006), as well as microsatellite-instability status could be an independent predictor of FU-based adjuvant chemotherapy (Ribic

et al., 2003). Furthermore, topoisomerase I expression has been investigated as a predictive factor for response to IRI (Paradiso *et al*., 2004), and high mRNA expression of excision repair cross-complementing 1 and thymidylate synthase have shown to be predictive of poor response to treatment with oxaliplatin and FU in advanced disease (Shirota *et al*., 2001).

Recently, the combined analysis of genetic polymorphisms in TS, uridine diphosphate glucutonyltransferase and X-ray cross complementing factor 1 genes was demonstrated to be a prognostic factor able to predict a better time to progression in patients with advanced CRC treated with FU combined with OHP or IRI (Martinez-Balibrea *et al*., 2007). Furthermore, some genetic phenotypes have been validated as potential predictive factors of IRI and/or FU pharmacokinetics. Indeed, UGT1A1*28 polymorphism has been associated with a decreased glucuronidation of SN38 and an increased toxicity with this agent (Toffoli *et al*., 2006), whereas the GSTT1-null genotype may be useful in predicting toxicity in response to IRI/FU regimens (Romero *et al*., 2006).

However, although predictive factor testing is an exciting field of research, it has not yet been applied routinely in clinical practice. An *in vitro* study on prediction of response of CRC cells demonstrated that the selection of multiple genes which best correlated with FU-induced apoptosis may predict response more effectively than four previously established determinants of FU response: thymidylate synthase and thymidine phosphorylase activity, and p53 and mismatch repair status (Mariadason *et al*., 2003). In such perspectives, Del Rio *et al*. (2007) evaluated the gene expression profile of primary CRC tissues and identified a set of 14 genes that could predict the response to FOLFIRI. The major application of these studies would be to use the gene signature as a decision tool to assist oncologists in selecting CRC patients who could benefit from chemotherapy, avoiding ineffective and toxic therapies. Indeed, this is even more crucial for adjuvant treatment, for which the rationale is to reduce the rate of tumor recurrence and mortality in patients who have undergone curative surgery.

FUTURE DIRECTIONS

Major progress has been achieved in the clinical management of CRC patients. These results have been obtained by both the advent of novel anticancer agents and by the optimization of drug regimens. Indeed, a significant number of studies has been performed to improve the clinical activity of traditional chemotherapeutic schedules and presently such regimens achieve response rates of 50–60% and overall survival of ~ 20–22 months in the advanced disease (Venook, 2005). By contrast, the understanding of the interactions between molecular-targeted agents and chemotherapeutic drugs is far to be complete. While initial studies revealed very exciting results, a major goal of preclinical and clinical research in this field is to improve our knowledge in the clinical use of these novel biological agents and in the optimization of their use in combination with traditional chemotherapeutic drugs.

In a different perspective, another objective of clinical research is to find molecular tools able to predict the resistance to specific anticancer agents and, thus, avoid useless and potentially toxic treatments. While the evidence already obtained has

not yet been applied to daily clinical practice, the availability of new molecular biology technologies, able to study thousands of gene at the same time, may likely provide significant improvements in the near future. Indeed, pharmacogenomic studies are expected in CRC patients to select subsets of genes able to predict the sensitivity of tumor cells to specific agents or to specific schedules, providing clinical oncologists the tool to personalize anticancer treatments.

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8
Detection of Abdominal Abscesses After Colorectal Surgery: Ultrasonography, Computed Tomography and Gallium Scan

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INTRODUCTION

Despite advances made in surgical techniques, antibiotics use and supportive care in recent years, abdominal infection due to an anastomotic leakage remains one of the most dangerous complications following colorectal surgery. The current trend for surgical management of colorectal cancer has changed from conventional open approach to minimal invasive laparoscopic colectomy (Finlayson and Nelson, 2005). The anastomotic leakage rate after colon resection has been reported from 2% to 15% of cases (Soeters *et al*., 2002; Rullier *et al*., 1998) and has not been reduced with the new laparoscopic technique when compared with open surgery (Breukink *et al*., 2005). A delay in the diagnosis and treatment of an abdominal abscess may result in sepsis and multiple organ system failure, a common cause of death in postoperative patients. Therefore, early detection of a colorectal anastomotic leak and abdominal abscess, followed by adequate treatment, is crucial and can significantly reduce mortality.

RISK FACTORS

Many factors may increase the risk of colorectal anastomotic leakage and result in abdominal abscess. Studies have shown that individual surgeon's skill is the most important risk factor (Stumpf *et al*., 2005; Alves *et al*., 2002). Other surgical factors in relation to a successful colorectal anastomosis include the maintenance of adequate blood supply to both anastomotic ends, accurate apposition of the seromuscular coats, tension-free construction of the suture, meticulous hemostasis, and avoidance of anastomosis in the presence of peritonitis.

In addition to the aforementioned factors, other risk factors which may increase the risk of colorectal anastomotic leakage are as follows:

- Malnutrition: A body weight loss more than 10–15% in the 6 months prior to the operation or the presence of hypoproteinemia increases the risk of anastomotic leakage (Mäkela *et al*., 2003; Soeters *et al*., 2002).
- Cardiopulmonary disease: Patients with underlying cardiopulmonary problems

have higher surgical risk and hence, higher incidence of anastomotic leakage after colorectal surgery (Ansari *et al*., 2000).

- Lifestyle: Smoking and alcohol consumption are associated with colorectal anastomotic leakage. Sorensen *et al*. (1999) reported that patients who smoke or drink in excess of 35 standard drinks per week have a significantly higher risk of developing an anastomotic leakage than those who do not smoke or drink after colorectal surgery.
- Site of anastomoses: The risk of anastomotic leakage reduces as the anastomosis locates more proximally. Rudinskaite *et al*. (2005) reported that the risk of anastomotic leakage is 3.5 times higher for anastomoses situated at or 10 cm distally from the anal verge than those situated 10 cm proximally from the anal verge. Similarly, Rullier *et al*. (1998) found that anastomoses situated at or below 5 cm from the anal verge are 6.5 times more likely to develop a leakage than those situated higher than 5 cm from the anal verge.
- Gender and obesity: Studies have shown that male and obese patients have higher risk of colorectal anastomotic leakage. Walker *et al*. (2004) reported an anastomotic leakage rate of 6.3% for male patients compared with 2.9% for female patients (p value < 0.05) after colorectal surgery. Benoist *et al*. (2000) found that the anastomotic leakage rate was 16% for obese patients and 6% for nonobese patients after colorectal resection and the difference was statistically significant.

CLINICAL DIAGNOSIS

The symptoms of a leaked colorectal anastomosis range from mild temperature elevation to fecal discharge in the wound. Despite the variation in its clinical presentation, early detection of an anastomotic leakage and abscess formation is still possible by close monitoring and frequent clinical examination. An anastomotic leakage or abscess formation should be considered if any of the following signs is observed in a patient after colorectal surgery:

- Unexplained fever with leucocytosis, tachycardia or malaise.
- Adynamic ileus or pelvic pain.
- Fecal discharge: fecal discharge from wound, drainage tract, rectovecical or rectovaginal fistula.
- Peritonitis or septicemia.
- Cardiac complication: Cardiac complications such as postoperative chest pain, dyspnea and peripheral edema following colorectal surgery should raise the suspicion of an anastomotic leakage. Sutton *et al*. (2004) reported that 43% patients who have anastomotic leakages after colorectal surgery presented with seemingly unrelated cardiac symptoms instead of the typical symptoms and signs of peritonitis. In this cohort, the diagnosis of abdominal abscesses was delayed by up to 11 days in patients with cardiac complaints and such delay was a major cause of death.

The anastomotic leakages are classified into two categories: major and minor. The clinical manifestations of a major leakage are those of peritonitis and septicemia. A minor leakage is accompanied by less dramatic clinical signs or no signs of sepsis. For an experienced surgeon, the diagnosis of a major leakage after colorectal surgery is not difficult to make. However, a minor leakage may be more difficult to diagnose because the typical symptoms and physical findings, such as abdominal pain and tenderness, may be obscured due to recent abdominal surgery and distorted

anatomy (Lin *et al*., 2002). In addition, many of the clinical indices for infection, such as body temperature measurement, peripheral white blood cell (WBC) counts or serum C-reactive protein (CRP), are often elevated as part of the normal physiological response during the postoperative period. In these situations, one or more imaging methods are necessary to obtain a rapid and correct diagnosis.

CLINICAL MANAGEMENT

Once an anastomotic leakage has occurred following colorectal surgery, every effort should be made to contain the leakage and resulting sepsis in order to reduce the morbidity and mortality.

A major anastomotic leakage following colorectal surgery causes extravasation of large amount of fecal fluid in the peritoneal cavity and almost always results in peritonitis and overwhelming sepsis. An aggressive surgical approach is necessary as these complications are potentially fatal (Ansari *et al*., 2000). If the anastomotic leakage is combined with a large adjacent abscess, internal or external drainage of the abscess is usually performed, followed by appropriate antibiotics treatment. A laparotomy with the intention of constructing a diverting colostomy or Hartmann procedure may be necessary at times to properly evacuate the abscess (Soeters *et al*., 2002). Treatment failure is mainly due to inadequate removal of infected fluid or tissue.

A minor leakage following colorectal surgery is accompanied by less dramatic clinical signs. These patients can be managed conservatively with antibiotics and total parenteral nutrition. However, these patients require close monitoring and surgery should be carried out immediately in cases of acute deterioration or sepsis (Soeters *et al*., 2002).

ABDOMINAL RADIOGRAPHY

The conventional radiography has been reported as an imperative method in the initial detection of abdominal abscesses, particularly in the subphrenic region and the upper abdomen. However, abdominal radiographs may not be ideal in the diagnosis of lower abdominal abscesses, because the abscesses may be difficult to detect in the pelvis or between bowel loops due to superimposition of adjacent shadows. Only 13–50% of patients with lower abdominal abscesses have abnormal findings on plain abdominal radiographs (Fry *et al*., 1980). The contrast radiographic study provides additional diagnostic information in the detection of abdominal abscesses. This method enhances the definition of intra- or extraluminal gas pattern to demonstrate the displaced or compressed gut lumen. In addition, bowel wall perforation or suture line leakage into the abscess cavity can also be demonstrated by intraluminal contrast medium. Unfortunately, contrast radiographic studies following colorectal surgery offer little advantage in predicting the early post-operative morbidity compared to the plain abdominal radiographs. There is no indication to advocate the use of routine contrast enema because a radiological leak does not alter clinical management in the majority of cases (Akyol *et al*., 1992). Furthermore, some complications, such as perforation, have been reported after contrast radiographic study, especially in patients who had recent lower gastrointestinal surgery. Therefore, conventional radiography is seldom the

sole basis upon which a clinical decision for reoperation or drainage is made.

ULTRASONOGRAPHY

Ultrasonography is a useful diagnostic tool in the detection of abdominal abscesses and has been recommended as the first line of investigation because it is noninvasive, relatively inexpensive, easy to perform with no radiation exposure, portable and the results can be known immediately. Ultrasonography is highly operator dependent. Its sensitivity in detecting an abdominal mass ranges from 40–50% to more than 90% in experienced hands (Knochel *et al*., 1980). Ultrasonography is particularly useful in demonstrating perihepatic and intrahepatic collections because the liver is a good sonographic window. Similarly, a urine-filled bladder serves as a good sonographic window for the detection of pelvic collections. Unfortunately, this technique is less sensitive in the left upper quadrate and mid abdomen region because the ribs and the gas-filled bowel loops may scatter the sound beam. The diagnostic findings of an abdominal abscess on ultrasonography include a round, oval or elliptical collection of fluid with varying degrees of wall thickness and the abscess fluid is of various homogeneous echogenicities, depending on the stage of the abscess.

There are a number of shortcomings associated with ultrasonography in the detection of abdominal abscesses in the early postoperative patients. Any object that prevents good contact of the ultrasound probe with the abdominal wall, such as surgical dressings, suture lines, open wounds, drainage sites, and stomas may hinder the performance of sonography. Furthermore, the distorted anatomical relationships of the early postoperative patients can further obscure the diagnosis of abdominal abscesses. The performance of sonography may be even more unsatisfactory in patients who just had colorectal surgeries for the following reasons: (1) These patients usually have significant ileus, and the presence of abdominal abscesses can further exacerbate ileus. Abscesses located behind the gas-filled bowel loops are particularly difficult to diagnose with ultrasonography. In addition, a fluid-filled bowel loop may occasionally be mistaken for an abscess. (2) Abdominal ultrasonography is more effective in detecting abscesses that are located in the right upper quadrant and the pelvis. However, abscesses secondary to colorectal anastomotic leakages can be formed in other abdominal regions and are less likely to be detected by ultrasonography. In a study by Lin *et al*. (2005), they found the sensitivity of ultrasonography was 42.9% and false-negative rate was 57.1% for detecting abdominal abscesses after colorectal surgery. The results indicate that ultrasonography may be less useful than first thought in detecting abdominal abscesses after colorectal surgery.

COMPUTED TOMOGRAPHY

Computed tomography (CT) has many advantages for the diagnosis of abdominal abscesses.

1. Computed tomography scans take short duration, usually less than a minute, to perform. This may be of major importance for critically ill patient.
2. Computed tomography scans can extend from the diaphragm to the symphysis

pubis. It covers the entire abdomen without the interference from underlying bone or bowel gas. It is particularly helpful in the postoperative patients in whom ultrasound examinations are difficult to perform because of the presence of interfering objects, such as surgical dressings, suture lines, drainage sites and stomas.

3. Computed tomography provides a high image resolution and clearer identification of anatomical details with respect to the size and the location of an abscess so that safe routes for diagnostic aspiration or therapeutic drainage can be planned. CT also shows good differentiation of abscess and phlegmonous change.
4. Computed tomography is most useful for demonstrating fluid collections within the abdominal cavity and is highly specific for the diagnosis of abdominal abscesses. In a study by Koehler and Moss (1980), CT correctly excluded the presence of abscess in 31 of 32 cases (specificity 97%). Additionally, this method is capable of detecting incidental findings unrelated to the abscess that may affect postoperative management such as pseudomembranous colitis, diverticulitis, cholangitis, and cystitis.
5. Computed tomography is a suitable imaging modality for obese patients since the low attenuation of adipose tissue provides an ideal contrast for other tissues.

Techniques and Imaging Findings

Conventionally, the abdomen is scanned in 8 or 10 mm thickness at 16 or 20 mm increments from just above the diaphragm to the symphysis pubis. Spiral CT is performed with a 8 or 10 mm collimation and scan pitch factor of 1–2. The definition of pitch is used as "the distance table traveling per rotation/the thickness of collimation". The sensitivity of CT scan is related to the thickness of each abdominal slice. A thinner CT slice provides a better imaging resolution and is more sensitive in the detection of abdominal abscesses. However, a thinner CT slice may increase the radiation exposure due to prolonged scanning time. Therefore, there is a trade-off between a clearer imaging resolution and radiation exposure.

The administration of oral or intravenous contrast medium further aids the diagnosis of abdominal abscesses on CT scans. The use of diluted oral contrast medium prior to scanning enables the clinicians to differentiate fluid-filled intestinal loops from abscesses or other fluid collections. In addition, contrast medium may identify extravasations, which usually indicate the presence of fistulas or suture line failures. However, patients may not tolerate the administration of a large volume oral contrast, nor can the contrast medium pass through to the distal bowel efficiently after colorectal surgery. The use of intravenous contrast medium is another method to aid the diagnosis of abdominal abscesses on CT scans. Intravenous contrast medium may significantly enhance the appearance of abscesses on CT scans by concentrating the contrast material around the pus collection, without the disadvantages of oral contrast medium discussed above (Fry, 1994).

The CT appearance of abdominal abscesses varies, depending on their stage of evolution. Initially, a nonspecific soft tissue inflammation or a phlegmon may be visualized. As the abscess ripens, its center undergoes liquefaction and the surrounding wall thickens. It progresses into

a unilocular or multilocular cystic mass with thin or thick, well-defined or irregular wall, which appears as multiple fine bubbles or as a single large bubble on abdominal CT scan (Figure 8.1). In general, there are five major CT signs for an abscess: (1) a well-defined soft tissue mass with a low density center (2–29 HU); (2) abnormal gas pattern within the mass, either as a mottled pattern of small bubbles or with one or more air-fluid levels. Up to 50% of the abdominal abscesses have air bubbles within its center on CT scans (Fry, 1994); (3) displacement or compression of the surrounding viscera due to the mass effect of the abscess; (4) the ring sign, seen as a peripheral rim enhancement surrounding the abscess after intravenous administration of contrast agent. This is due to increased flow of contrast agent through the dilated inflamed vessels and interstice in the abscess wall; and (5) edema of the surrounding tissue.

Utility

Computed tomography scan is highly efficacious in the diagnosis of abdominal abscesses, with accuracy greater than 95%. Eckmann *et al*. (2004) reported an accuracy of 96.7% for CT scan in the detection of anastomotic leakage following low anterior resection in patients with rectal cancers. In patients after colorectal surgery, CT scan can also detect the presence of abdominal abscesses with a high degree of accuracy. Lin *et al*. (2005) evaluated 23 patients with suspected abdominal infection after colorectal surgery and reported an accuracy rate of 95.7% for CT scan in diagnosing abdominal abscess. In another study of 34 patients after colorectal surgery, Tsai *et al*. (2001a) reported accuracy for CT in the detection of abdominal abscesses was as high as 97.1%. In addition, CT scans has the added advantage of concurrently demonstrating an abdominal wound

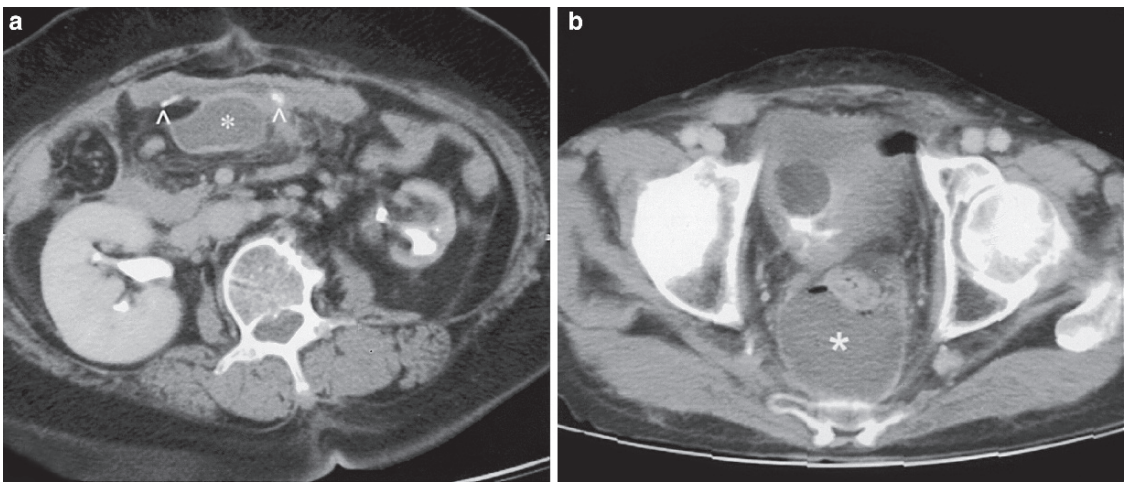

FIGURE 8.1. (a) An abscess in the peritoneal cavity after right hemicolectomy for cecal carcinoma. Computed tomography scan after contrast administration shows a loculated fluid collection (asterisk) with an enhanced rim and edema of the surrounding tissue. Anastomotic clips are noted (arrowheads). (b) The CT scan shows a well-defined abscess (asterisk) with a peripheral rim of enhancement and small air bubbles behind the sigmoid colon after anterior resection for sigmoid colon carcinoma

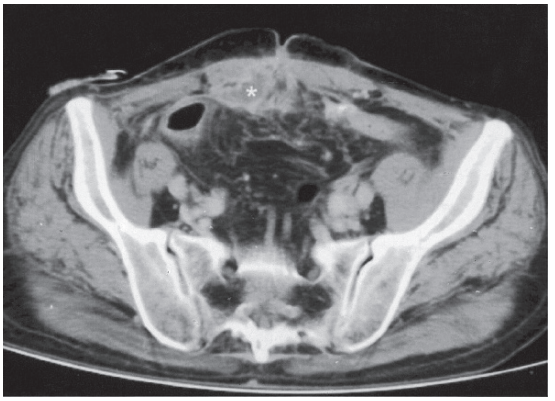

FIGURE 8.2. Computed tomography scan shows an abscess (asterisk) with a peripheral enhancement in the anterior abdominal wall and edema of the surrounding tissue near the surgical wound after right hemicolectomy for ascending colon carcinoma. This patient was diagnosed with deep wound infection

infection while searching for an abdominal abscess in patients after abdominal surgery (Figure 8.2).

Although CT is considered the best available imaging modality in the evaluation of abdominal abscesses after colorectal surgery, it is not without problems. First, CT scan is unable to differentiate an infected fluid collection from an uninfected one without needle aspiration or drainage, since the densities of these fluid collections are similar. Differential diagnoses of an abdominal fluid collection include pseudocysts, cysts, urinomas, lymphoceles, bilomas, old hematomas, and necrotic tumors. Nevertheless, gas within the mass is virtually pathognomonic of an abscess. Second, the quality of the CT image can be radically degraded by the presence of metallic surgical clips, which may induce "sparkler" diffraction artifact. Third, in patients after abdominal surgery, the distorted anatomy may hamper the interpretation of CT image.

False-negative results have been reported for CT studies in the evaluation of abdominal abscesses. In a retrospective analysis, Jasinski *et al.* (1987) found that the sensitivity of CT scan varies depending on the location of the abscesses, from 60% in the interloop region to 100% in the right subphrenic area, lesser sac and retroperitoneum. Three systemic errors were identified to account for the 15 false-negative cases in their study. The technical error can result from excess patient movement and poor bowel opacification after radiopque contrast administration. The interpretative error is due to the failure of the physician to locate the abscess on CT scan because the abscess resembles a loop of bowel or other diseases. The observer perceptive error is due to the failure of the physician to locate the abscess that can be seen in retrospect, such as reader fatigue. In addition, false-negative result can occur in an early infection, before the development of a discrete fluid collection, particularly in patients with distorted anatomy due to recent abdominal surgery. False-positive cases are usually due to misclassifying an uninfected fluid collection as an abscess. However, this rarely poses a serious problem because the reported false-positive rate is low. Dobrin *et al.* (1986) reported a specificity of 93% for CT in the diagnosis of abdominal abscess. Two recent studies have both reported the specificity of 100% for CT scan in the evaluation of abdominal abscesses after colorectal surgery (Lin *et al.*, 2005; Tsai *et al.*, 2001a).

Roche (1981) reported that the diagnostic information obtained from CT studies significantly modified the clinical management in over 55% of patients. However, CT scans may not always assist the clinical decision-making. In a study of critically ill surgical patients with suspected abdominal

infection, Norwood and Civetta (1985) found that no CT scan was reported positive within 8 days after the abdominal surgery, and the diagnostic information from 50 of 72 CT scans was of no help or not used in the clinical management of these patients. Lal *et al*. (2000) successfully developed an evidence-based guideline to optimize the timing and use of CT studies in patients with suspected abdominal abscesses. This guideline recommended that a CT study should only be performed if the patient meets the following criteria:

1. The patient is postoperative or has abdominal pain and tenderness, or both.
2. The patient must have fever, elevated white blood cell count, or bacteremia.

In the current cost conscious health care system, the appropriate use of CT studies in patients with suspected abdominal abscesses after abdominal surgery is an important consideration in the clinical management of these patients.

GALLIUM-67 CITRATE SCAN

Gallium-67 citrate (gallium) scan was originally developed as a tumor-imaging modality. It was soon applied to the evaluation of tissue inflammation and became the first radionuclide method widely used in this field. Gallium scan is relatively inexpensive but the delay in the diagnosis and the interference from intense physiologic bowel activity are major drawbacks which limit its use in the investigation of abdominal pathologies. The development of computed tomography (CT) and magnetic resonance imaging (MRI) has dramatically improved the way clinicians diagnose abdominal abscesses. They provide an accurate and immediate diagnosis. For these reasons, CT and MRI have supplanted nuclear medicine scans for the evaluation of patients with suspected abdominal abscesses. Most studies have shown that CT is the imaging method of choice for the diagnosis of abdominal abscesses (Tasi *et al*., 2001b; Dobrin *et al*., 1986). However, the presence of an abdominal abscess is significantly more difficult to detect by CT in patients with distorted anatomy due to recent trauma or surgery or with an early infection before a discrete fluid collection is developed. In these clinical situations, radionuclide scanning may be of some value. Moreover, in patients without localizing signs of an abdominal abscess, nuclear medicine imaging still plays a significant role because of its high sensitivity and the ability to survey the entire body.

Normal Biodistribution and Mechanisms of Localization

Gallium is an element in group IIIA of the periodic table and acts biologically as an iron analog. After intravenous administration, gallium is rapidly bound to transferrin and haptoglobin. Because of the high affinity to protein, its plasma clearance is relatively slow. Approximately 10% of the gallium remains in the plasma 24 h after the intravenous administration. Approximately 10–25% of the injected gallium is excreted by the kidneys, predominantly within the first 24 h. Another 10% of the dose is excreted into feces in two ways. The majority (80%) is directly transported across the intestinal mucosa; a small portion (20%) is excreted via the hepatobiliary route (Datz, 1996). The liver metabolizes both transferrin and lactoferrin, causing significant gallium uptake. Physiological uptake of gallium in the salivary glands, nasal region, skeletal system and spleen

can also be seen on the gallium scan. Mild to moderate lung uptake is frequently seen between 6 and 24 h after gallium injection but diminishes after that. Radioactivity in the urinary system is normal on the first day after gallium injection, but is considered pathological if such activity is visualized after 48- hours. Colonic activity, especially in the ascending and transverse colons, is often prominent in delayed images. This can cause a problem in the interpretation of the abdominal scans. Often, serial scans are required to correctly interpret the abdominal activity.

Various mechanisms have been proposed to explain the accumulation of gallium in inflammatory lesions (Ando *et al*., 1990). Gallium may enter the intercellular space of the inflammatory tissue due to increased permeability and stay there by binding to the acid mucopolysaccharide present in the inflamed tissue. Some bacteria and other pathogenic microorganisms may take up gallium directly via siderophores. In addition, gallium may bind to the lactoferrin in blood leukocytes that migrate to inflammatory sites. However, the exact mechanism is still not completely understood. It is likely that one or more of the above mechanisms result in the gallium uptake by the infective lesions.

Instrumentation and Technique

Generally, a dose of 185 MBq (5 mCi) of gallium is recommended for imaging the inflammatory lesions in adults. Proportional reduction of the dose is suggested for pediatric patients on the basis of the 185 MBq dose to a 70-kg adult. A gamma camera with a medium-energy, parallel-hole collimator is usually used to obtain the images. Three 20% windows set at 93, 184 and 296 keV are suggested. The entire body should be scanned because an unsuspected infectious site may be detected (Tsai *et al*., 2001b). A single photon emission computed tomography (SPECT) imaging, which provides three-dimensional information and has a better delineation of the abnormal gallium uptake, can be obtained depending on the clinical indication.

If the purpose of the gallium scan is to detect the presence of a tumor, images are usually taken 48–72 h after gallium injection. However, such delay may be too long for a patient with a suspected infective focus. For patients with suspected abdominal abscesses, earlier imaging after gallium injection has been recommended. Abdominal imaging can be performed as early as 4–6 h after gallium injection, during which the predominant route of gallium excretion is still the genitourinary system. However, a 6-h image may encounter the following problems: (1) There is usually not enough time to perform a 6-h image if the patient is referred to the Department of Nuclear Medicine after midday. (2) The background to target ratio may be low in a 6-h image due to high gallium concentration in the plasma. Perkins (1981) reported that approximately 50% of patients scanned between 5 and 9 h after gallium injection had an increased physiological accumulation of gallium, which can be confused with pathological activity. If an early 4–6 h image is not available or is inconclusive, a routine 24-h image is recommended (Tsai *et al*., 2001b).

The normal gastrointestinal excretion of gallium can limit its usefulness in the diagnosis of abdominal lesions. In some patients, it is difficult to differentiate physiological bowel activity of gallium from an abdominal infection and false-positive result may occur (Dobrin *et al*., 1986). Two methods have been proposed to reduce the interference of normal gallium bowel activity during scan interpretation: (1) serial imaging,

(2) the use of cathartics and enemas. Images taken at 48, 72 h or even later may be necessary and useful for distinguishing normal bowel activity of gallium from an abdominal infection. The location of normal gallium activity in the bowel changes with time, whereas an infective lesion or abscess remains static. The use of cathartics has been suggested to remove as much gallium as possible from the colon prior to imaging. Unfortunately, studies have shown conflicting results with regard to the usefulness of bowel preparation prior to gallium scintigraphy. A study by Zeman and Ryerson (1977) showed that intensive bowel preparation, involving the administration of three 5 mg bisacodyl tablets for three consecutive nights after gallium injection and 360 ml of magnesium citrate orally the night before the scan was taken, did not reduce colonic gallium activity significantly when compared with control group. Silberstein *et al*. (1981) concluded that milk of magnesia and cascara did not significantly speed up the removal of gallium from the intestine or improve the scan quality. On the contrary, Novetsky *et al*. (1981) used four regimens of bowel preparation and found that castor oil was most effective in cleansing the bowel activity before the gallium scintigraphy. According to our experience, the accumulation of gallium in the hepatic flexure and splenic flexure of the colon can be significantly reduced using either castor oil or Bisacodyl (Hsieh *et al*., 2000). However, cathartics and enemas may not be suitable in patients who had recent colorectal surgeries and shall be used with caution.

Utility

Gallium scan has been used for the detection of abdominal abscesses for decades. The reported sensitivity and specificity of gallium imaging for the detection abdominal infection varies. Biello *et al*. (1979) reviewed the literature and reported that gallium scintigraphy has a sensitivity of 91% and a specificity of 93% for the detection of abdominal abscesses. Other investigators have reported a somewhat lower accuracy (Dobrin *et al*., 1986). High false-positive rate, as high as 22%, for gallium scan in the evaluation of abdominal abscess has been reported in the literatures, and is predominantly due the to normal bowel excretion of gallium (Datz, 1996). As discussed earlier, serial imaging is usually required to distinguish the normal bowel activity from a real abdominal abscess (Tsai *et al*., 2001b). However, such differentiation can be difficult to make at times. Static gallium activity in the abdomen is usually indicative of an infective source but other possibilities cannot be excluded. Many patients with suspected abdominal abscesses have reduced peristalsis, meaning that static gallium activity may be due to normal bowel activity. In a study of 61 patients who underwent colorectal surgeries and subsequently developed fever of unknown origin, Tsai *et al*. (2001b) have reported two false-positive cases due to prolonged gallium excretion in the colon: one had increased gallium activity at the hepatic flexure and the other one at the splenic flexure. The intensity of gallium uptake in the colon was equal to the liver uptake in both cases and did not shift nor change pattern for as long as 3 days after gallium injection.

The use of gallium scan is more problematic in postoperative patients due to increased gallium uptake as the result of post-surgical inflammation, tissue damage or wound infection. Wound infection is a common source of postoperative fever and

causes significant morbidity and sometimes mortality. The incidence of postoperative wound infection varies, depending on the surgeon, the medical institution, and even the surgical procedure. The overall wound infection rate ranges from 1.8% to 9.4% (Weiss *et al*., 1999; Yalcin *et al*., 1995), but can be as high as 32.1% after colorectal surgery (Yalcin *et al*., 1995). Therefore, the possibility of a wound infection shall always be kept in mind when interpreting a gallium scan. Knowledge of the normal gallium uptake in a clean surgical incision is essential before one can properly diagnose a wound infection on gallium scan. In our study evaluating the gallium uptake in clean surgical incisions after colorectal surgery, 61.5% of patients showed increased gallium uptake in the surgical wound and 23.1% showed even higher gallium activity at the incision site compared to the liver within 7 days after the surgery. Fifty percent of patients had increased gallium uptake at the surgical incision and 25% had higher gallium uptake than that in the liver 8–14 days after the surgery. Fourteen days after surgery, 12.5% of patients showed increased gallium uptake in the clean surgical wound but none had gallium intensity greater than that in the liver (Lin *et al*., 2001). Although increased gallium uptake is commonly seen in a clean surgical wound, it is still possible to differentiate a clean wound from an infected one on gallium scan based on the gallium uptake intensity and pattern. The gallium uptake of an infected wound is more intense than that in the liver or equal to the liver uptake combined with an irregular edge. Using this diagnostic criterion, Tsai *et al*. (2002) reported a diagnostic sensitivity of 94.4% and a specificity of 96.9% for gallium scan in the diagnosis of wound infection.

A lateral view is usually useful and sufficient to distinguish gallium uptake in a wound infection from an abdominal infection (Figure 8.3) (Tsai *et al*., 2002). A SPECT imaging is also helpful for such differentiation. However, the scanning time for an SPECT image is much longer than

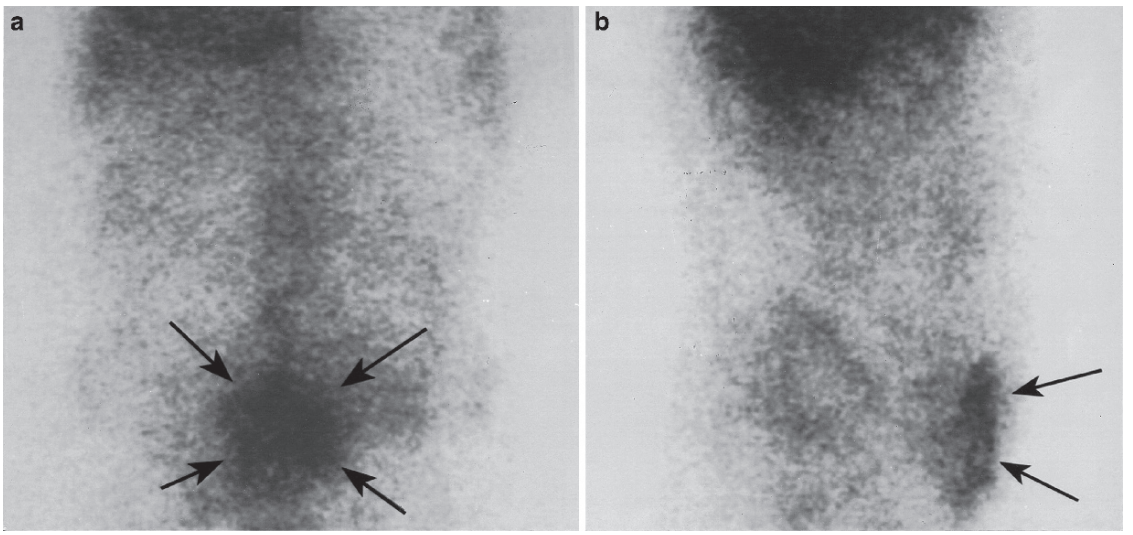

FIGURE 8.3. (a) The anterior view of the 24-h gallium image shows an area of increased gallium uptake in the low abdomen (arrows). (b) A lateral view confirmed the gallium activity is confined to the abdominal wall (arrows). The patient was diagnosed with a wound infection

that of a lateral view. In addition, the participation of the surgeons in gallium scan interpretation can further aid the diagnosis of an abdominal infection as clinical information in relation to the location of surgical wounds, colostomy, and the method of operation are crucial for such diagnosis. In our experience, the sensitivity, specificity and accuracy of gallium scan are 100%, 95.2% and 96.7% in the detection of an abdominal infection after colorectal surgery when surgeons are involved in the gallium scan interpretation based on the following criteria: an area of gallium uptake with an intensity equal to or greater than that of the liver and remains unchanged in the sequential images (Figure 8.4) (Tsai *et al*., 2001b).

The incidence of false-negative cases in the detection of abdominal abscesses is much lower than that of false positive cases. The sensitivity of gallium scan for abdominal abscesses detection has been reported to be higher than 90% in many studies (Biello *et al*., 1979; Moir and Robins, 1982). Subphrenic abscesses and large masses with secondary infection, such as hematomas and pseudocysts, are major causes for false-negative result. A subphrenic abscess is often missed due to the normal tracer uptake in the liver and spleen (Moir and Robins, 1982). However, the false-negative rate of gallium scan is less of a concern when used to detect the presence of abdominal abscesses after colorectal surgery. Two recent studies found no false-negative case when using gallium scan to detect the presence of abdominal abscesses after colorectal surgery (Lin *et al*., 2005; Tsai *et al*., 2001b). This is partly due to the fact that subphrenic abscesses or secondarily infected large hamartomas or pseudocysts of short

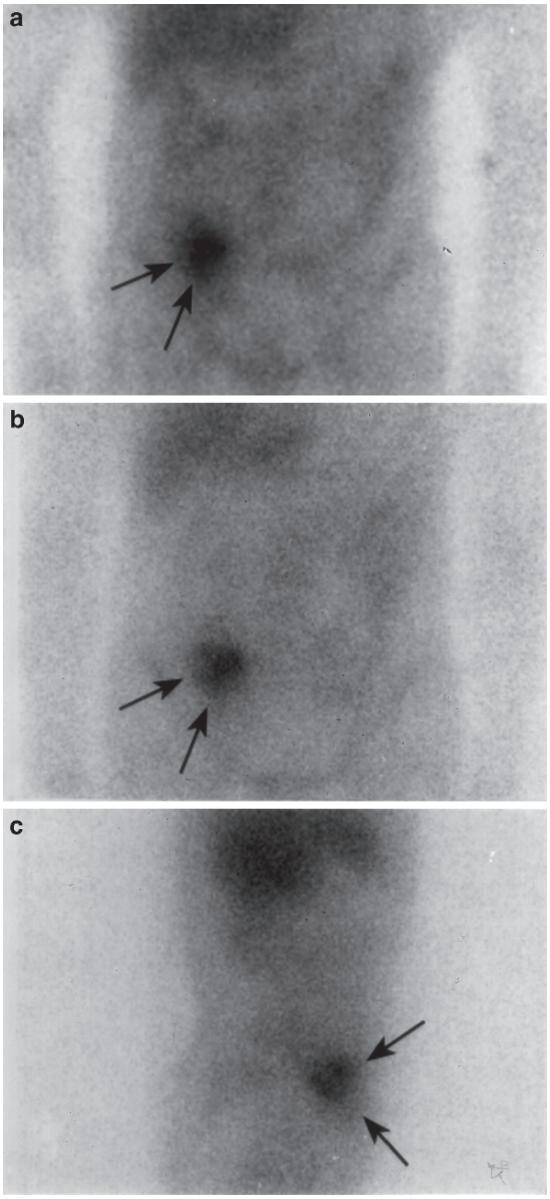

FIGURE 8.4. Gallium images in a patient with fever of unknown origin after colorectal surgery (a) The anterior view of the 24-h gallium image shows an area of increased gallium uptake with intensity in the right lower abdomen (arrows). Note the intensity of this gallium uptake is higher than that of liver uptake. (b) This gallium-avid lesion in the right lower abdomen did not change in shape on the sequential anterior view of the 72-h gallium image (arrows). (c) The lateral view of the 72-h gallium image shows the lesion is in the peritoneal cavity. The patient was diagnosed with an abdominal infection

evolution, the common causes of false-negative result for gallium scan in the detection of abdominal abscesses, rarely occur in patients who had recent colorectal surgeries.

In aging patients, the detection of an infective process is sometimes difficult since the clinical manifestations of infection are frequently subtle or atypical. The detection of a postoperative infection can be even more difficult in the elderly. Many of the typical findings of infection, such as fever, elevated peripheral blood WBC count or CRP, may be part of the physiological response during the postoperative period. The effectiveness of the gallium scan, CRP, WBC counts and body temperature in the detection of an infective process was evaluated in a study of 33 patients aged 60 years and over, with fever of unknown origin after recent colorectal surgery (Lin *et al*., 2002). Both WBC and body temperature measurement showed low sensitivity for detecting a post-surgical infection in the elderly population. The CRP test had good sensitivity but low specificity. In comparison, gallium scan had the best diagnostic accuracy. Furthermore, gallium scan has the added advantage of surveying and evaluating the whole body, not just the abdomen, during the diagnosis of a suspected abdominal abscess. Hence, other unexpected extra-abdominal infective sources can be identified. This is particularly important for patients with post-surgical fever but without any focal signs of infection.

Gallium Imaging Versus CT Imaging

It is well documented that CT scan has better diagnostic accuracy than gallium scan in the detection of abdominal abscesses. In the study by Moir and Robin (1982), the sensitivity and specificity for CT scan in the detection of abdominal abscesses were both 100% while gallium scan had a lower accuracy with a sensitivity of 96% and a specificity of 65%. In a recent study, the diagnostic values of gallium scan and CT scan in the detection of abdominal abscesses were compared in 34 patients after recent colorectal surgery. The overall accuracy, sensitivity and specificity for CT scan were 97.1%, 93.7% and 100%, respectively whereas gallium scan had a diagnostic accuracy of 91.2%, sensitivity of 100% and specificity of 95.2% (Tsai *et al*., 2001a).

OTHER RADIONUCLIDE METHODS

Leukocyte Scan

The recent development of leukocyte scan, using leukocytes labeled with indium-111 (In-111) oxine or technetium-99 m (Tc-99 m) hexamethylpropylene amine oxide (HMPAO), has supplanted gallium image for the evaluation of suspected intra and extra abdominal infections (Datz, 1996; Palestro *et al*., 2000). Labeled leukocytes provide a faster and more reliable diagnosis. In-111–labeled leukocyte scan is more suitable than Tc-99 m HMPAO labeled leukocyte scan or gallium scan in the evaluation of abdominal infection. In-111–labeled leukocyte, unlike gallium or Tc-99 m–labeled leukocyte, is not generally present in the normal gastrointestinal tract. In contrast, normal bowel excretion of gallium and Tc-99 m HMPAO labeled leukocyte can interfere with the imaging interpretation of

an abdominal abscess. Mountford *et al*. (1990) compared In-111 labeled leukocyte scan with Tc-99 m HMPAO leukocyte scan in the detection of abdominal abscesses. They found that both scans had a sensitivity of 100% but the specificity for Tc-99 m HMPAO labeled leukocyte scan was much lower than that of In-111 labeled leukocyte scan, mainly due to the physiological bowel uptake of Tc-99 m HMPAO labeled leukocyte. Although leukocyte scan has a better accuracy rate than that of gallium scan, its clinical application is limited due to the following reasons. In-111 is not available in many countries, and the labeling of Tc-99 m HMPAO with leukocytes is expensive and time consuming. In addition, the accuracy of leukocyte scan decreases in chronic infective lesions. In a study comparing In-111 leukocyte scan and gallium scan in the diagnosis of occult sepsis, imaging with In-111 labeled leukocyte was found to be more accurate for the diagnosis of an acute infection of short duration, whereas gallium imaging was superior for a protracted or chronic infection (Sfakianakis *et al*., 1982). Therefore, gallium image is still the preferred radionuclide method for the detection of abdominal infection in situations where leukocyte imaging is not available or if the suspected infection failed to incite a neutrophil response.

Radiolabeled Antigranulocyte Monoclonal Antibodies

Radiolabeled monoclonal antibodies (MoAb) against granulocyte antigens is an alternative technique that permits specific in vivo labeling of leukocytes without the disadvantages of in vitro labeling. Studies of large series of patients with suspected acute appendicitis showed not only rapid targeting of an affected appendix by the radiolabeled monoclonal antibodies (often within minutes), but also a very high negative predictive value of the test (Rypins *et al*., 2002). This technique has been reported to be useful in the detection of abdominal abscesses and other infections (Vicente *et al*., 2004). Radiolabeled MoAb scintigraphy may be a good alternative in centers where the facility for *in vitro* white blood cell labeling is not available.

Radiolabeled Human Polyclonal Immunoglobulin G

Another approach to the imaging of abdominal inflammation is with the use of In-111 or Tc-99 m labeled polyclonal human immunoglobulin G (IgG). Rubin *et al*. (1989) imaged 128 patients with In-111 labeled polyclonal IgG and found a sensitivity of 91% and a specificity of 100% for intra- and extra-abdominal infections. Radiolabeled polyclonal IgG is easy to prepare and has an excellent performance in a variety of clinical conditions, including the detection of suspected infection in immunocompromised patients. However, this radiopharmaceutical is not available in many countries and a multiple day imaging protocol is usually necessary due to the slow clearance of the injected IgG from the body.

CONCLUSION

In general, abdominal ultrasonography is the first line of investigation if an abdominal abscess is suspected on clinical ground

because it is relatively inexpensive, can be performed at bedside for severely ill patients and provides a rapid result. However, our experience shows that ultrasonography plays a minor role in the detection of abdominal abscesses after colorectal surgery. Most studies agreed that CT is the imaging method of choice for the diagnosis of abdominal abscesses, especially when there are localizing symptoms or signs. In addition, percutaneous drainage of the abscess is possible under CT guidance. We have the same recommendation for patients with suspected abdominal abscess after recent colorectal surgery. If an abdominal abscess is not visualized on CT scan but an early or an occult infection is still suspected on clinical ground, a gallium scan or other radionuclide images such as leukocyte scan should be performed to aid the diagnosis. The radionuclide scan is particularly useful in the absence of localizing signs and in cases of occult sepsis or fever of unknown origin. In conclusion, there is not a single imaging technique which is ideal for all patients. Computed tomography and nuclear medicine techniques offer additional information in the investigation of infective sources in patients after colorectal surgery.

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9
Antimetastatic Therapy in Colorectal Cancer: Role of Tumor Cell Matrix Metalloproteinase 9 (Methodology)

Wilhelm J. Lubbe and Giovanni M. Pitari

INTRODUCTION

Metastasis of tumor cells to distant organs is promoted by matrix metalloproteinases (MMPs) which cleave extracellular matrix and allow cancer cells to migrate and invade neighboring tissues. In colorectal tumors, a member of this family of enzymes, MMP-9, regulates key processes underlying metastasis, including cell adhesion, spreading, migration, invasion and angiogenesis. Recent studies have revealed that MMP-9 is highly expressed in colorectal tumor cells from patients and confers metastatic potential to cancer cells. Indeed, by signaling at the interface between the cell surface and the basement membrane in the pericellular environment of colon cancer cells, MMP-9 degrades extracellular matrix components, promotes cytoskeleton remodeling associated with cell spreading and, most notably, supports the ability of cancer cells to hematogenously seed distant sites of metastasis. These observations suggest that targeting tumor cell MMP-9 may be a novel highly specific approach to inhibit metastasis formation in patients with colorectal cancer.

In this chapter, key mechanistic dynamics underlying colorectal cancer metastasis will be presented because they represent the context for pathological MMP-9 functions. The mode of regulation of MMP-9 and the role of MMP-9 in distinct processes underlying metastasis of colon cancer cells will be analyzed. Also, strategies to target MMP-9 produced by tumor cells will be discussed, including the use of pharmacological inhibitors, genetic manipulation of MMP-9 expression and specific approaches targeting molecules regulating MMP-9 signaling. Emphasis will be devoted to specific methodologies for the study of the function of MMP-9 produced by colon cancer cells. These studies will establish the utility of targeting MMP-9 signaling in colon cancer cells to suppress metastasis formation and progression.

COLORECTAL CANCER METASTASIS

Colorectal cancer is the third most common neoplasm in the U.S. (Jemal *et al*., 2004). It is the second leading cause of cancer-related mortality, responsible for 10% of cancer-related deaths in the U.S. and worldwide. The mortality rate for large bowel cancer, ~ 50%, reflects metastases:

~ 20% of patients have metastatic disease at presentation and ~ 33% develop metastases during the course of their disease (Jemal *et al*., 2004). Unfortunately, current adjuvant chemotherapy for metastatic colorectal cancer increases median survival only ~ 14 months (Meyerhardt and Mayer, 2005). These observations highlight the need for rationally designed interventions to manage the metastatic process in patients with colon cancer.

Metastasis is the spread of tumor cells from the primary neoplasm to distant organs, and their progressive growth (Fidler, 2003). To date, the most successful therapeutic approaches are those targeting early-stage disease, before tumor cells have invaded and colonized extraintestinal sites. These include "curative" surgery intended to remove all clinically detectable tumor, and chemoprevention, which blocks colon cancer carcinogenesis employing pharmacological or dietary agents. A major problem for these interventions is residual micrometastases that result in relapse (Virgo *et al*., 1995). Also, iatrogenic dissemination of cancer cells as a result of surgical manipulation is an adverse outcome in colon cancer (Paraskeva *et al*., 2006). Recurrence rates vary widely, from 3% for disease limited to the intestinal mucosa to > 50% for tumors which have spread to regional lymph nodes (Virgo *et al*., 1995). Overall, ~ 50% of surgically treated patients suffer recurrent disease. Loco-regional recurrence occurs in ~ 30% of cases while distant recurrence occurs in ~ 80% (Virgo *et al*., 1995; Jemal *et al*., 2004). Thus, pharmacological interventions targeted to prevent, interrupt, or reverse metastatic progression of colon cancer may have an enormous impact on patient management.

Brief History

Arguably, the oldest documented cases of multiorgan metastatic cancer is represented by nine mummies of pre-Columbian Incas (Peru, dated 500 B.C.) possessing several metastatic lesions in their bones as a result of primary skin melanomas. For several centuries, the prevailing doctrine considered metastasis an entirely random process. This dogma remained uncontested until 1889, when the British surgeon Stephen Paget proposed the 'seed and soil' hypothesis of cancer metastasis.

Paget theorized that metastasis occurred in a nonrandom fashion, governed by intrinsic characteristics of both "the seed" (the cancer cell) and "the soil" (the distant organ): "When a plant goes to seed, its seeds are carried in all directions, but they can only live and grow if they fall on congenial soil" (Paget, 1989). Three years after Paget's death (1926), James Ewing, professor of pathology at Cornell University and co-founder of the American Cancer Society, largely dismissed "the seed and soil" theory suggesting that metastasis is exclusively determined by mechanical forces. He argued that the specific anatomy of the vascular system, but not the intravasated cancer cells, is the only determinant of metastatic dissemination. Ewing's theory lingered as the dominant model for decades.

A reevaluation of Paget's theory of metastasis was published during the 1950s by the laboratory of Dale Rex Coman. Using an experimental animal model, Coman's group demonstrated that cancer cells injected directly into the vasculature produced metastasis in some organs but not in others. From this group, Irving Zeidman demonstrated by microcinematography that only embolized tumor cells with rod-like

shapes arrested and formed metastasis, but not those rounded and flaccid. The predictive factor for metastasis was a physical property of the tumor cell, its rigidity, which forced it to be entrapped inside the microvasculature. Leslie Foulds extended these observations proposing that tumor cells undergo a transformation as they move closer to metastasis. This 'neoplastic progression' consists of the gradual development of automony by the tumor cells from the host through the acquisition of intrinsic and self-supporting properties conferring upon them the metastatic ability.

In the 1970s, fundamental support for Paget's seminal observations came from the work of Weiss and Sugarbaker, which demonstrated that while regional metastasis could be predicted on the basis of the vascular anatomy, distant organ metastasis was exclusively predicted by the type of primary neoplasm, independent of the anatomy of the circulation. More importantly, in 1984 David Tarin conclusively demonstrated that the critical factor in the formation of distant organ metastasis is the biological match of complementary traits possessed by both the traveling cancer cell and the distant organ. Thus, patients with advanced ovarian cancer experienced daily inoculation of millions of tumor cells into the circulation from palliative peritoneal-jugular vein shunt. Amazingly, they were not significantly affected by metastatic disease at the primary drainage organ, the lung, but continued to have peritoneal metastases at a similar rate compared to patients without shunt.

Mechanisms of Pathogenesis

Metastatic colon cancer cells have as their privileged distant target organs, the liver and the lung. To reach their final destination, these cells have to perform a multistep process encompassing invasion of the intestinal wall, detachment from surrounding stroma, intravasation into blood vessels, embolization, distribution, and seeding of distant tissues. Of intravasated colorectal tumor cells, ~ 0.1% (~ 0.02% of cells in solid tumors per day) remain viable after 24 h, and > 99.99% are eliminated before reaching their sites of metastasis. The inefficiency of tumor cell embolization and distribution reflects the biological and genetic heterogeneity of the primary colorectal cancer, which contains only a few subpopulations of cells with metastatic attributes, including the ability to invade, spread, migrate, and resist apoptosis (Fidler, 2003). These cells derive from the clonal evolution of cancer stem cells within the primary tumor, and express unique phenotypic characteristics promoting invasion and survival. Following distribution, these select subpopulations of cells seed metastatic distant sites by adhering to the endothelial surface of blood vessels, proliferating, and invading tissue parenchyma (Al-Mehdi *et al*., 2000; Wang *et al*., 2004). Moreover, enduring metastases require resistance to local immune defenses, establish sustaining interactions with the host microenvironment, and develop an autonomous vascular network (Fidler, 2003).

Mechanisms leading to colorectal cancer metastasis are selective and require the orderly sequence of several rate-limiting steps. Thus, while metastatic progression has an impact on the prognosis of patients, mechanisms underlying invasion and metastasis are vulnerable and offer unexploited possibilities for targeted therapy. Indeed, interventional strategies that disrupt the metastatic cascade could reduce mortality

from colorectal cancer and permit long-term management of patients with primary tumors. At the basis of these approaches is the identification of molecular targets selective for the metastatic process. Among these, the activity of MMP-9 is emerging as a key regulator of the malignant phenotype.

MATRIX METALLOPROTEINASE 9 (MMP-9)

Matrix metalloproteinases (MMPs) are an expanding family of zinc-dependent metalloendopeptidases that regulate cell locomotion, growth and angiogenesis, and play essential roles in metastasis (Coussens *et al*., 2002). The first evidence of the activity of MMPs originated from the observation that proteolytic enzymes were responsible for the dissolution of the tadpole tail. Thus far, more then 20 human MMPs have been discovered and labeled with numerical designations, reflecting the order of their identification. Also known as matrixins, MMPs belongs to the superfamily of metzincins, which include serralysins, adamalysins, astracins, and ulilysin. The metzincins share a consensus sequence of three histidines in the catalytic domain that form the zinc-binding site. All MMPs exhibit three conserved domains: a secretory signal sequence, a prodomain, and a catalytic domain bearing the consensus sequence for the zinc-binding site.

The catalytic activity of these endopeptidases can degrade all protein components of the extracellular matrix (Stamenkovic, 2003). Indeed, on the basis of this classical matrix-degrading ability and their substrate specificity, MMPs have been divided into collagenases, gelatinases, stromelysins, and matrilysins. However beyond extracellular matrix remodeling, MMPs can also regulate growth factors and their receptors, adhesion molecules, cell surface proteoglycans, chemokines, cytokines, and a myriad of enzymes (Stamenkovic, 2003). Notably, these effects are, in part, independent from their catalytic activity, further underscoring the function of MMPs as complex signaling molecules of the extracellular space. This is reflected in their critical role in several (patho)physiological processes, including metastasis, wound-healing, inflammation, and tissue remodeling (Coussens *et al*., 2002). In particular, metastasis of colorectal cancer cells to distant organs, such as the lung and liver, is initiated and promoted by the secretion of MMPs, most notably MMP-9, which degrades basement membrane, permits cell migration and invasion, and regulates tumor growth (Zeng *et al*., 1999; Yu and Stamenkovic, 2000).

Function and Regulation of MMP-9

The MMP-9 cDNA was first cloned from transformed human fibroblasts and shares > 80% homology with murine, lupine, and bovine transcripts (Atkinson and Senior, 2003). The human MMP-9 gene is located on chromosome 20q11.1–13.1, and contains Ets and NF-kB binding sites. Similarly, AP-1, AP-2, and SP-1 binding sites all exist within the 2 kb 5' flanking region of the MMP-9 promoter, permitting unique modes of MMP-9 regulation in different cell systems and biological conditions. Various endogenous factors regulate the synthesis or release of MMP-9, such as tumor necrosis factors and interleukins (Yao *et al*., 1997).

In humans, MMP-9 is synthesized as a preprozymogen and secreted as a catalytically inactive 92-kDa proenzyme (Atkinson

and Senior, 2003). Thus, the structure of MMP-9 consists of a propeptide sequence conserved amongst all MMPs, and fibronectin type II-like repeats shared by MMP-9 (gelatinase B/92-kDa type IV collagenase) and MMP-2 (gelatinase A /72-kDa type IV collagenase) (Atkinson and Senior, 2003). The fibronectin type II-like repeats exist within the catalytic domain of the enzyme and enhance the binding affinity of MMP-9 to the extracellular matrix components gelatin and elastin (Shipley *et al*., 1996). Although MMP-9 and MMP-2 degrade many of the same extracellular matrix components, distinct regulatory domains impose specific characteristics. Indeed, MMP-9 specifically interacts with vascular endothelial-derived growth factor (VEGF) and degrades tissue basement membrane, playing a key role in angiogenesis and tumor invasion (Zeng *et al*., 1999). In addition, MMP-9 uniquely possesses a collagen type V-like domain that alters its substrate specificity and may render the enzyme resistant to degradation (Atkinson and Senior, 2003).

Generally, MMP-9 expression correlates with tissue remodeling and invasion in (patho)physiological processes (Matsubara *et al*., 1991). Once released, proMMP-9 is activated by cleavage of the 10-kDa N-terminal domain. An array of proteases and factors can activate MMP-9, including in descending order of potency MMP-3 (stromelysin), MMP-2 (gelatinase A), and hypochlorous acid. Also, MMP-1 (interstitial collagenase), plasmin, and thrombin bind to and allosterically activate MMP-9. Conversely, signaling by MMP-9 is inhibited by various factors, including α_2-macroglobulin, which complexes with and clears circulating MMPs, and thrombospondins and tissue factor protease inhibitor-2 (TFPI-2), which bind to and inactivate MMP-9. However, the most specific inhibitors of MMP-9 catalytic activity are the tissue inhibitors of matrix metalloproteinase (TIMPs). Among these, TIMP-1 binds to the carboxyl terminus of the proenzyme as well as to the catalytic domain of the active enzyme, neutralizing its activity by (1) forming a non-covalent complex with secreted MMP-9, (2) preventing formation of MMP-9 homodimers or MMP-9/MMP-1 heterodimers, and (3) inhibiting MMP-3-dependent MMP-9 activation as a TIMP-1/MMP-9/MMP-3 complex.

Signaling of MMP-9 in Metastasis

Release and activation of MMP-9 are prerequisites for its ability to degrade the extracellular matrix. However, MMP-9 can also support signaling by mechanisms distinct from its catalytic activity. Thus, proMMP-9 expression is associated with tumor invasion *in vitro* (Huang *et al*., 2001). Inhibition of proMMP-9 binding to integrin $\alpha_V\beta_3$, but not its catalytic activity, prevents the growth of HSC-3 tongue squamous cell carcinoma xenografts in nude mice *in vivo* (Bjorklund *et al*., 2004). In addition, inhibiting MMP-9 expression, but not enzymatic activity, suppressed cell migration, and induced cytoskeleton reorganization and E-cadherin-mediated cell-cell adhesion in Ewing's sarcoma cells (Sanceau *et al*., 2003). The proposed mechanism for these latter effects was the ability of proMMP-9 to directly associate with the surface adhesion molecule CD44 on cancer cells (Sanceau *et al*., 2003). In this model, CD44 acts as a scaffolding molecule that facilitates cancer cell adhesion, invasion and angiogenesis by specifically assembling functional units at the cell surface comprising MMP-9, extracellular

soluble factors, and intracellular cytoskeletal proteins.

The growth, intravasation and metastasis of tumor cells are largely influenced by MMP-9 signaling. Indeed, MMP-9 also is termed the 92-kDa type IV collagenase because this activity is important for degrading type IV collagen in basement membrane, liberating invasive cancer cells from their epithelial compartment (Harvey *et al*., 2003). In colorectal cancer, beyond its role in matrix-degradation, MMP-9 promotes cancer cell metastasis by regulating multiple rate-limiting steps of the metastatic process. Transcriptional and post-transcriptional activation of MMP-9 has been linked to early mutational events during colorectal tumorigenesis, suggesting a role for this gelatinase in cancer cell proliferation (Leeman *et al*., 2003). Indeed, MMP-9 activity is required for optimal DNA synthesis by colon carcinoma cells *in vitro* (Agrez *et al*., 1999). In addition, MMP-9 activity contributes to tumor-driven neovascularization and growth. Tumor angiogenesis is essential for both metastatic dissemination of cancer cells and establishment of enduring metastasis in host tissues. In that context, MMP-9 positively regulates angiogenesis, in part, by specifically activating and releasing transforming growth factor and VEGF from the cell surface and the extracellular matrix, respectively (Yu and Stamenkovic, 2000).

Furthermore, MMP-9 regulates two fundamental and complementary processes of colorectal cancer cell metastasis: forward extension of invadapodia and detachment from surrounding stroma. Invadapodia are the locomotory units of invading cancer cells, driven by polymerization of the actin cytoskeleton which extends the cell membrane into pseudopodia in single cells or lamellipodia in multiple coordinated cells. MMP-9 accumulates at the leading edge of pseudopodia in invading endothelial cells during angiogenesis (Nguyen *et al*., 1998), supporting a crucial role for this gelatinase in cell invasion. Also, MMP-9 activity is important for invasion through the basement membrane of both normal and tumor cells (Sanceau *et al*., 2003). Conversely, to invade, colorectal cancer cells must detach from their associated stroma. By specifically degrading type IV collagen, MMP-9 disrupts bonds between cell adhesion molecules and the basement membrane in focal adhesion complexes. As a result, there exists a dynamic interplay between adhesion molecules and MMP-9, which regulates the invasive phenotype of tumor cells. In fact, reciprocal regulation between MMP-9 and $\alpha_v\beta_6$ integrins induces a metastatic phenotype in colon cancer cells (Agrez *et al*., 1999). Similarly, the adhesion molecule CD44 facilitates cancer growth, invasion and angiogenesis, in part, by specifically binding MMP-9 (Yu and Stamenkovic, 2000).

After the extracellular matrix has been digested, cancer cells spread through the cleared extracellular space. Cell spreading drives migration and requires the regulation of adhesion to the extracellular matrix and cytoskeleton remodeling. The functional unit of spreading is the formation, in the direction of movement, of pseudopodia/lamellipodia, which are transiently, but firmly, attached to the extracellular matrix by labile contacts, enabling cytoskeleton anchorage and subsequent traction-driven migration. In lung epithelial cells, MMP-9 regulated lamellipodia adhesion to collagen IV through these labile contacts and MMP-9 inhibition completely prevented cell spreading and migration (Buisson *et al*., 1996).

In addition, MMP-9 activity promoted migration in several other cell types, including macrophages, lymphocytes, keratinocytes, and tumor cells. Thus, MMP-9 provides colorectal cancer cells with unique attributes which enhance mobilization and may promote their extraintestinal relocation.

Tumor Cell MMP-9

An emerging model suggests that MMP-9 in colorectal cancer cells mediates the formation of metastatic colonies in target tissue parenchyma. Thus, inhibitors of MMP-9 suppressed colorectal metastases in rat liver (Aparicio *et al*., 1999). Similarly, infusion of the MMP-9 inhibitor TIMP-1 with melanoma cells reduced metastases in mice (Schultz *et al*., 1988). Conversely, inhibition of TIMP-1 expression increased metastasis of BALB 3T3 cells (Khokha *et al*., 1989). These observations indicate that MMP-9 may mediate crucial steps following tumor intravasation required for metastatic spread *in vivo*. Indeed, forced MMP-9 expression induced a metastatic phenotype in transformed rat embryo cells (Bernhard *et al*., 1994). Moreover, tumor cells transiently expressing MMP-9 generated significantly more metastases than parent MMP-9-null cells in a mouse model of lung metastasis. Because metastases often were devoid of MMP-9, it was suggested that MMP-9 may specifically subserve early phases in the seeding process, including extravasation, adhesion, and invasion (Bernhard *et al*., 1994).

A controversial point is represented by the cellular source of MMP-9 that signals in colon tumor epithelial cells. The current paradigm suggests that stroma surrounding cancer cells is the principal source of MMP-9 in colorectal tumors (Collins *et al*., 2001; Roeb *et al*., 2001). Although MMP-9 expression increases in colorectal tumors compared with matched normal tissues from patients, studies suggest that tumor cells do not express MMP-9 (Roeb *et al*., 2001). Rather, soluble factors released by tumor cells induce expression and secretion of MMP-9 from stromal fibroblasts (Roeb *et al*., 2001) and tumor-infiltrating inflammatory cells (Collins *et al*., 2001). Thus, in the context of the extracellular matrix composition of the host tissue, fibroblasts and inflammatory cells, most notably macrophages, provide cancer cells with unique abilities essential to metastatic progression. In this model, tumor cells exploit physiological mechanisms for their metastatic dissemination because stromal cell MMP-9 plays crucial roles in processes such as inflammation and wound healing.

However, recent studies have demonstrated that human colorectal cancer cells *in vitro* express MMP-9 mRNA and protein, and release catalytically active MMP-9 into the media (Lubbe *et al*., 2006). Notably, this MMP-9 regulates the metastatic behavior of colon cancer cells, including their ability to degrade extracellular matrix components, form locomotory organelles and spread, and hematogenously seed mouse lungs (Lubbe *et al*., 2006). In these studies, well-differentiated colon cancer cells which express MMP-9 were employed, compared with poorly differentiated cell lines that did not release functional MMP-9 employed in earlier studies (Collins *et al*., 2001; Roeb *et al*., 2001). In addition, tumor cells isolated from patients by laser capture microdissection express MMP-9 mRNA, and they express MMP-9 protein using transmission electron microscopy (Lubbe *et al*., 2006). This is in

contrast to earlier studies where expression of MMP-9 could not be detected in human colon tumors by immunohistochemistry or Northern blot hybridization. In fact, laser capture microdissection permits precise collection of homogenous populations of cells from tissue and the subsequent quantification of MMP-9 mRNA by RT-PCR, while transmission electron microscopy allows the identification of intracellular structures otherwise undetectable by light microscopy. In these analyses, MMP-9 was expressed equally by cancer and stromal cells within colon tumors, which exhibited higher MMP-9 levels compared to epithelial or stromal components from normal mucosa (Lubbe *et al*., 2006). These results are particularly significant because they reveal the potential of targeting MMP-9 expressed by tumor cells for treating patients with colon cancer.

TARGETING MMP-9 IN COLORECTAL CANCER METASTASIS

The critical importance in mechanisms underlying metastasis suggests that MMP-9 could be a useful therapeutic target in patients with colon cancer. Surprisingly, MMP-directed therapies generally have failed in suppressing metastasis and improving clinical outcome (Coussens *et al*., 2002). These disappointing results have been principally ascribed to the lack of focused experimental models on MMP-mediated metastasis, and the unavailability of selective MMP inhibitors targeting processes promoting tumor metastasis. Another obstacle to MMP-based antimetastatic therapies may be the widespread perception in the MMP field that fibroblast and macrophages of the tumor stroma express MMP-9, rather than cancer cells that seed distant organs. However, MMP-9 mediates metastatic progression by signaling in the pericellular environment of cancer, and not stromal cells (Fridman *et al*., 2003). In addition, the stromal reaction to tumor formation may represent a potentially protective antitumor host-defense mechanism, which would explain the conflicting results obtained in clinical trials with therapies employing nonselective MMP inhibitors. Indeed, general MMP-directed therapies that are otherwise untargeted with respect to the source of enzyme may abrogate those critical adaptive antitumor mechanisms. The precise elucidation of MMP-9 biology and molecular mechanisms regulated by this gelatinase underlying colorectal cancer metastasis certainly would help the progress of antimetastatic approaches targeting MMPs.

Detection of MMP-9 in Tumor Cell Compartments

The molecular and functional analysis of MMP-9 in colorectal tumors may become a valuable prognostic tool for patient management. Since MMP-9 promotes metastatic disease progression, quantification of the levels of that gelatinase in the primary tumor could suggest the appropriate clinical follow-up. Indeed, MMP-9 is often overexpressed in colorectal tumors, and this dysregulated production may be associated with higher metastatic risk (Lubbe *et al*., 2006). Importantly, transformed epithelial cells with metastatic seeding abilities express functionally active MMP-9 (Lubbe *et al*., 2006) mediating early, rate-limiting metastatic steps, including tissue invasion, intra- and extravasation (Bernhard *et al*., 1994). Thus,

characterization of the cellular complement of MMP-9 in colorectal tumors may be of great value for novel anti-metastatic, MMP-9-based therapies. In that regard, surgical specimens from patients with colorectal adenocarcinomas should be interrogated following optimal handling and processing techniques to preserve tissue and cell biology, and molecular integrity. To permit comparative evaluation and functional assessment, analyses should be done on both the adenocarcinoma and normal adjacent tissue as confirmed by histopathology. As discussed above, determination of MMP-9 expression and activity in discrete tumor cell compartments is important because MMP-9 in tumor epithelial cells may mediate different biological processes than in mesenchymal cells of the reactive stroma, with opposing impacts on metastasis.

The expression of MMP-9 can be studied by quantifying the mRNA or protein content in clinical samples. For detection of MMP-9 mRNA, the reverse transcriptase polymerase chain reaction (RT-PCR) may be employed. A more rapid version of this technique, the real-time RT-PCR, permits reverse transcription, amplification, detection, and quantification of the target mRNA in one step by online fluorescence detection, using degenerate specific primers and a sequence-specific dual-labeled fluorogenic hybridization probe (Lubbe *et al*., 2006). To allow intra- and inter-patient comparisons, mRNA levels should be normalized to total RNA or a housekeeping gene. In contrast, the protein content of MMP-9 may be visualized by employing immunoblot analysis of total cell protein extracts or immunohistochemistry of tissue slides. An array of anti-MMP-9 antibodies may be used for these assays, including those directed against the pro-peptide domain, the hinge region, or the catalytic portion of MMP-9.

Quantification of MMP-9 expression in discrete cell compartments may be achieved by laser capture microdissection (Lubbe *et al*., 2006). This technique entails the collection of pure cell populations by using a focused low intensity laser as ultraprecise scalpel. Cryostat-sectioned flash frozen tissue samples are mounted on an inverted microscope and cell isotypes are captured by laser microdissection. An increased resolution of the cell complement of MMP-9 could be obtained by immunoelectron microscopy, which permits the visualization of proteins at the subcellular compartments providing the MMP-9 topography within specific cell types (Figure 9.1) (Lubbe *et al*., 2006). Finally, the activity of MMP-9 in clinical specimens may be studied by assessing the matrix-degrading ability of this protease through quantification of its gelatin-cleaving activity. Functional assays commonly employed in these studies include (1) gelatin zymography that identifies the catalytic activity associated with both the pro- (92 kDa) and active (82–86 kDa) MMP-9 form, and (2) gelatinase activity assay and (3) *in situ* zymography, which examine the matrix-corroding potential contained in tissue extracts or tissue slides, respectively, as a net result of the activity of MMP-9 and its endogenous inhibitors.

Reducing MMP-9 Expression in Cancer Cells

Colorectal cancer metastasis is greatly influenced by the activity of MMP-9. The finding that colon cancer cells express (Figure 9.1) and secrete MMP-9 promoting their metastatic dissemination (Lubbe

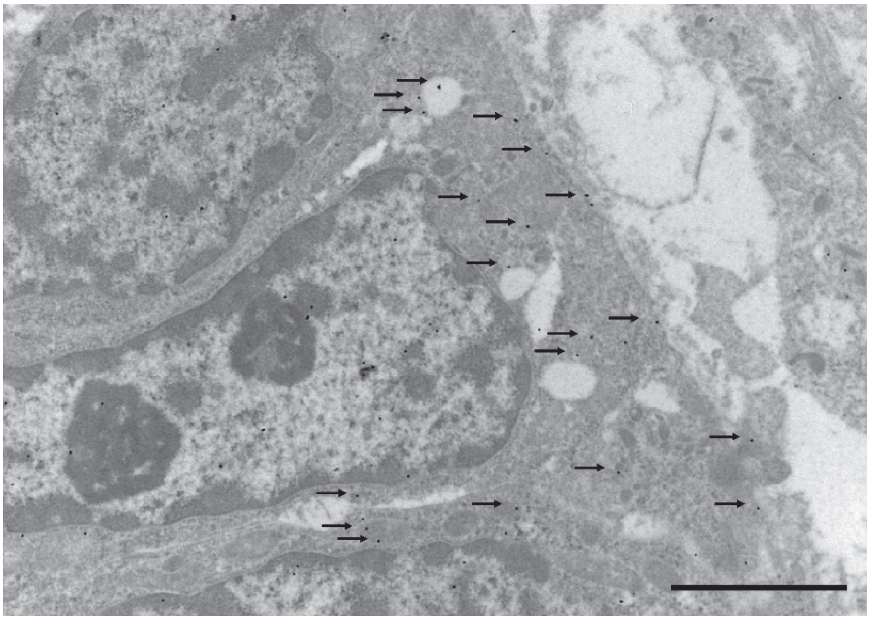

FIGURE 9.1. **Colorectal cancer cells produce MMP-9**. Image of colon carcinoma cells from patients by electron microscopy. Arrows indicate immunostaining for human MMP-9 at basal pole of cancer cells using a mouse monoclonal antibody visualized with a gold-labeled antimouse immunoglobulin G antibody. Bar, 2 μm

et al., 2006) forms the basis for tumor-selective therapies directed to disrupt the activity of this protease associated with metastatic progression. In that context, colorectal tumor epithelial cells, but not stromal cells, are the selective target of inhibition for novel chemopreventative strategies against colorectal cancer metastasis.

One approach for those specific therapies is to disrupt the ability of cancer cells to produce and release in the extracellular environment functionally competent MMP-9. Indeed, in order to fully exert its metastatic activity in the extracellular space, first MMP-9 has to be appropriately processed inside the cell, and then delivered at the invading microdomain in the cell surface membrane through regulated routes of intracellular trafficking. In theory, agents able to exert transcriptional, translational, or post-translational regulation of colon cancer cell MMP-9 may be exploited to impose significant reduction of this gelatinase at the functional membrane microdomain. Studies on cancer cells can be designed to explore the intracellular pathways of MMP-9 metabolism and discover novel targets for antimetastatic therapy. In particular, RT-PCR could be used to examine transcriptional regulation of MMP-9 expression by quantifying changes in mRNA. The contribution of translation to changes in MMP-9 protein expression could be examined by immunoblot analysis in the presence and absence of inhibitors of translation, including rapamycin.

Post-translational regulation of MMP-9 should be studied by focusing on two distinct but complementary processes determining the MMP-9 activity at the cell surface, including (1) the secretion of

MMP-9 from the cancer cells into the extracellular space, and (2) the internalization and intracellular degradation of secreted MMP-9 bound to adhesion molecules at the tumor cell membrane (Fridman *et al*., 2003). These studies often employ a combination of experimental strategies, including treatments of cells in culture, fixation and staining, confocal microscopy, and immunoblot analyses. Thus, internalization of MMP-9 has been explored in cells following ice-cold labeling of surface-associated MMP-9 with a monoclonal antibody and fixation, employing confocal microscopy to detect the internalized, antibody-bound MMP-9 visualized with the fluorescent secondary antibody (Arnaoutova *et al*., 2003).

Progress on molecular mechanisms underlying intracellular processing has already identified key regulators of the expression: membrane targeting, and secretion of MMP-9. Gene methylation affects the expression of MMP-9, and DNA methylation inhibitors increase MMP-9 mRNA and protein by reducing the methylation at CpG sites in the MMP-9 promoter (Chicoine *et al*., 2002). Also, MMP-9 expression is regulated by growth factors, inflammatory cytokines and adhesion molecules which affect its transcription or transduction. In this way, interleukin-1 and tumor necrosis factor-α induced MMP-9 transcription (Bergers *et al*., 2000), while α3β1 integrin promoted MMP-9 mRNA stability (Iyer *et al*., 2005). In contrast, platelet-derived growth factor signaling through phosphatidylinositol 3-kinase inhibited MMP-9 expression (Esteve *et al*., 2002). These observations suggest that therapeutic interventions aimed to interfere with the genomic methylation status or the signal transduction of external stimuli could have an impact on MMP-9 produced by cancer cells.

Intracellular trafficking, secretion and endocytosis of MMP-9 are critically influenced by the chemical and physical properties of microdomains at the cancer cell membrane, wherein signaling molecules regulate the function of MMP-9 in an outside-in manner. Thus, MMP-9 is regulated by gangliosides, integrins, focal adhesion kinase, and macromolecular units of growth factor receptor signaling (Zhang *et al*., 2006). These factors direct membrane targeting and release of MMP-9 on the basis of a complex interplay between cell metabolism, intracellular cytoskeleton, and extracellular matrix. In the context of tumor targeted therapies, activation of caveolin-1, a structural component of caveolae/raft microdomains present in enterocytes could prevent the release of MMP-9 from tumor epithelial cells (Williams *et al*., 2004). Moreover, the low density lipoprotein receptor-related protein, LRP, binds with high affinity to pro-MMP-9 at the cell surface and promotes internalization and degradation of the MMP-9/TIMP-1 complex (Hahn-Dantona *et al*., 2001), a mechanism whose induction would prevent MMP-9 signaling in the cancer pericellular space. Finally, MMP-9 secretion by mitogen-activated protein kinases MEK/ERK is prevented by inhibiting Raf-1 (Baccarini, 2005), a general signaling mechanism which may be targeted in different cell systems, including colon cancer cells.

To achieve the therapeutic goal of reducing colon cancer MMP-9 promoting metastasis, strategies may include non-pharmacological (e.g., genetic manipulations employing antisense or small interference RNA methodologies) or pharmacological (e.g.,

drugs acting as agonists or antagonists) approaches targeting a key mediator (e.g., NF-kB, integrin, caveolin, LRP) of the biological process (e.g., transcription, mRNA stability, secretion, endocytosis) involved in the synthesis, secretion or catabolism of MMP-9. Increased efficacy could be achieved by combination therapies inhibiting or inducing two or more of those pathways at the same time. Because the fundamental elements regulating MMP-9 expression only partially have been defined, the study of the specific molecular components contributing to the regulation of MMP-9 should reveal previously obscure signaling elements underlying neoplastic cell biology that could serve as novel markers and targets in colorectal cancer.

Suppressing MMP-9 Signaling in Cancer Cells

Another approach for cancer cell-specific antimetastatic therapy is to inhibit the signaling mechanism underlying metastasis regulated by MMP-9. As detailed above, MMP-9 promotes cancer progression by regulating multiple physiological steps amplified during metastasis including cell spreading and migration, matrix degradation, and invasion, intravasation, and interaction with adhesion molecules mediating organ seeding. Angiogenesis and proliferation underlying tumor neovascularization and growth at the metastatic site also are promoted by the catalytic activity of MMP-9 (Bergers *et al*., 2000; Yu and Stamenkovic, 2000). Each of these MMP-9-driven pathological processes, either alone or in combination, could become a target of novel therapies for patients with colorectal cancer. Studies in the laboratory can be designed that closely model the dynamics of those processes in patients. Although they cannot completely reproduce the human system, these experimental models have been instrumental in advancing our understanding on the function of MMP-9 and its interactions with other signaling molecules in cancer cells. Importantly, they may permit the identification of novel antimetastatic targets to be exploited in clinical applications.

Investigations that examine adhesion, migration and invasion by MMP-9 are very insightful. These processes underlie metastasis of cancer cells as a result of the classical function of MMP-9 in degrading macromolecular components of the extracellular matrix, allowing tumor cells to invade tissues. Tumor cell adhesion defines the ability of cancer cells to establish connections with tissue parenchymal components and colonize sites of distant metastasis. In this experimental model, tissue culture plates are coated with specific extracellular matrix components which are cleaved by MMP-9, including fibronectin, laminin, and types I and IV collagen. To prevent nonspecific binding, plates are usually incubated with bovine serum albumin before addition of cancer cells on the top of matrix-coated surfaces. Then, cell/matrix interactions are allowed to occur over the appropriate length of time, non-adherent cells are removed with a series of washing, and cancer cells firmly attached to the extracellular matrix are stained and quantified.

In contrast, cell migration and invasion study the ability of cancer cells to travel along two dimensional surfaces and penetrate tissue parenchyma, respectively, through ordered cycles of matrix erosion, detachment and spreading. These processes can be examined in colon cancer cells using

the transwell apparatus which consists of a set of two-chamber wells separated by pores of different size, with the distinction that for invasion a synthetic version of the extracellular matrix (e.g., matrigel) is typically added to the top chamber to mimic epithelial basement membrane. Cells are laid on the top chamber, a chemoattractant is added to the lower chamber, and migration or invasion is quantified as the bottom chamber/top chamber ratio of tumor cells that have moved through the pores over a fixed period of time (Sanceau *et al*., 2003).

Recent studies have demonstrated that MMP-9 produced by colon cancer cells promotes actin polymerization-driven locomotory organelle formation (Lubbe *et al*., 2006). They examined the fraction of cells that extend pseudopodia (cell spreading), membrane extension formed by the polymerization of the actin cytoskeleton, during a brief incubation period following cancer cell distribution onto multi-well plates (Lubbe *et al*., 2006). Cell spreading could also be complemented by direct visualization of actin polymerization dynamics by confocal microscopy, employing fluorescent probes that bind to actin (e.g., Alexa Flur 488 phalloidin).

Importantly, a novel *in vivo* model has been developed to explore the role of MMP-9 expressed by colon cancer cells in metastatic disease progression (Al-Mehdi *et al*., 2000; Lubbe *et al*., 2006). This model focuses on the process of hematogenous tumor cell seeding (Figure 9.2), the ability of metastatic cancer cells that have escaped the primary tumor to colonize target tissues following intravascular

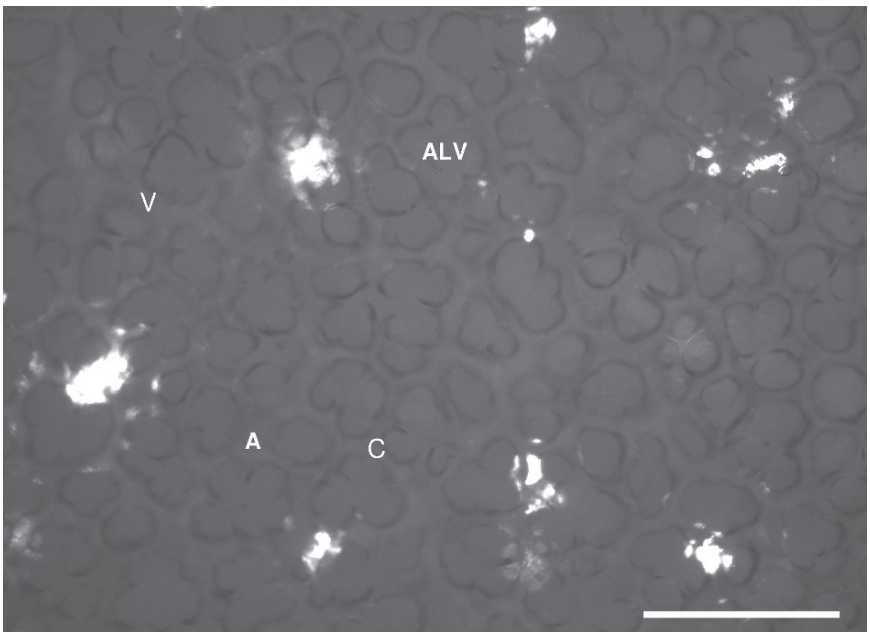

FIGURE 9.2. **Hematogenous seeding of mouse lung by human colorectal cancer cells**. Detail of a representative inverted fluorescence microscope field of the mouse lung parenchyma. Bright spots represent fluorescent cancer cells trapped in the subpleural vasculature of lung. A, arteriole; V, post-capillary venule; C, capillary; ALV, alveolus. Bar, 200 μm

adhesion. Because it is a necessary step toward metastasis formation, disruption of this rate-limiting process could prevent colon cancer spread to the lung or liver. Briefly, tumor cells are labeled with a vital fluorescent dye and injected into the inferior vena cava of anesthetized nude mice (with a compromised immune system), artificially ventilated through a tracheotomy. Five minutes after injection, lungs are cleared of blood, isolated and inflated with 5% CO_2 in air. Then, fluorescent tumor cells attached to pulmonary vessels are detected using an inverted fluorescent microscope (Figure 9.2), and quantified by scanning multiple consecutive fields from each lobe of the lung (Al-Mehdi *et al*., 2000). Employing this technique, tumor cell MMP-9 was demonstrated to mediate hematogeneous seeding of human colorectal cancer cells in lungs, specifically by affecting the earliest events underlying metastasis (Lubbe *et al*., 2006), which involve adherence to exposed basement membrane and cell spreading on vascular endothelial surfaces of parenchymal organs (Wang *et al*., 2004).

These results are particularly significant because they represent a prerequisite condition for mechanism-based, tumor-specific therapies targeting MMP-9 in patients with colorectal cancer metastasis. However, suppression of MMP-9 signaling is predicated upon inhibiting or inducing the activity of specific proteins regulating cancer cell MMP-9. Certainly, direct inhibition of the activity of MMP-9 itself would be the approach of choice. This could be achieved by delivery of small molecule inhibitors or gene-targeting constructs (e.g., small interference RNA) directly to the cancer cells. Alternatively, endogenous inhibitors such as TIMP-1 could be administered or locally induced in tumor cells to suppress MMP-9 functions in cancer cells. Similarly, upregulation of the reversion-inducing cysteine-rich protein with Kazal motif (RECK), a membrane-anchored glycoprotein with protease-inhibitor like domains downregulated in tumors, would sequester MMP-9 at the cell membrane and inhibit its activity (Takahashi *et al*., 1998). This protease almost exclusively signals at the interface between the invading tumor cell and the surrounding matrix, in close proximity to the cell membrane but in intimate functional relationship with the extracellular space (Fridman *et al*., 2003). In that context, processes underlying metastasis by MMP-9 are regulated by a complex interplay between tumor cell adhesion molecules, such as CD44 and integrins, MMP-9-activating macromolecular complexes, including the uPAR/uPA/plasmin/MMP-3 and MT1-MMP/MMP-2/MMP-13 axes, and extracellular matrix components, such as collagen I and IV (Fridman *et al*., 2003). It is reasonable to speculate that inhibition of any of those molecules in colon cancer cells would disrupt MMP-9 signaling and prevent metastasis in patients.

CONCLUDING REMARKS

Beyond its role in promoting the degradation of extracellular matrix components, MMP-9 has been involved in a variety of distinct, crucial processes underlying metastasis. Hence, MMP-9 regulates proliferation, growth, angiogenesis and invasion of tumor cells, including those originating from the colon. Importantly, this gelatinase affects key rate-limiting steps of the metastatic cascade, from adhesion to migration,

and from intravasation to extravasation. Consistent with the hypothesis that the function of MMP-9 is instrumental for tumor cell metastasis, increased levels of this gelatinase are positively associated with metastatic disease progression in colon cancer (Saito *et al*., 2000). Conversely, tumor cells from patients with hereditary non-polyposis colon cancer, which present constitutively lower MMP-9 activity, exhibit a less invasive phenotype (Moran *et al*., 2002).

Novel findings demonstrate that human colon cancer cells express high levels of MMP-9, which are comparable to those present in tumor stromal cells, the principle source of MMP-9 for colon tumors in the current paradigm (Lubbe *et al*., 2006). Characterization of the cellular origin and functional contribution of MMP-9 to colorectal tumor metastasis is important in defining the role of that protein in mechanisms underlying disease progression. Moreover, colorectal cancer mortality largely reflects the development of metastases (Jemal *et al*., 2004). Thus, preventing metastasis by specifically targeting tumor cell MMP-9-dependent processes represents a previously unrecognized therapeutic strategy that could significantly improve patient management in colorectal cancer. Unfortunately, a major obstacle to this therapeutic approach is the absence of selective inhibitors that target MMP-9 mediating metastasis of tumor cells, but not MMP-9 mediating physiological functions in normal tissues. One strategy to target cancer cell MMP-9 could be the use of fusing constructs between specific MMP-9 inhibitors and antibodies or ligands that selectively recognize antigens or receptors on cancer cell surfaces. Another successful approach may derive from the discovery of new molecules or receptors selectively expressed on colorectal cancer cells that specifically regulate cancer cell MMP-9, whose targeting will disrupt only MMP-9-dependent processes associated with metastasis. In this way, metastasis formation in patients with colorectal cancer could be suppressed without inducing unwanted side effects and collateral damage in normal tissues.

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10
Endoscopic Resection of Early Colorectal Tumors: Novel Diagnostic and Therapeutic Techniques

Paul Hurlstone, S. Brown, and Wal Baraza

EARLY COLORECTAL CANCER: A PARADIGM SHIFT

The adenoma-carcinoma model of colorectal cancer pathogenesis as described by Morson (1968) currently forms the basis of colonoscopic screening for colorectal cancer. Cellular genetic activity triggers morphological changes within the tissues which take on polypoid form. With increasing size, the likelihood of malignant change within the polyp also increases. The detection and histological analysis of these protuberant adenomas has been the mainstay of secondary cancer prevention in the West for the past 30 years. Their early detection and histological analysis direct various stratagem aimed at preventing progression to malignant disease. Studies on colonoscopic polyp screening suggest that the removal of exophytic lesions results in a higher than expected incidence of colorectal cancer. The National Polyp Study (Winawer *et al*., 1993) reported on the 6-year follow-up of 1,418 patients after repeated colonoscopy to clear all polyps. While this study did not have a true control arm, the background age and sex specific incidence of colorectal cancer was used as a control group. The findings were that the removal of all polyps seen prevented the development of 75% of carcinomas. The Veterans Affairs Study (Muller and Sonnenberg, 1995) found that only 50% of cancers were prevented, but the study was limited by the fact that not all patients had received total colonoscopy.

One possible factor responsible for polyp surveillance failing to prevent all colorectal cancers within these studies may be due to the lack of Western experience of flat and depressed lesions within the colon. These lesions may not be detected, recognized, or adequately treated and some of them may develop carcinomatous change. Indeed, such lesions were reported by Japanese researchers in the 1980s, but were thought to be irrelevant to Western populations and described as ‘phantom’ or Akita’s carcinoma. During the past decade, however, there have been efforts to establish an East–West consensus on the clinicopathological importance of macroscopic polyp morphology incorporating not only exophytic polyps but also flat and depressed lesions. Our group prospectively studied the prevalence and clinicopathological characteristics of flat and depressed colorectal lesions in a single UK-based cohort in patients at a high risk

of developing colorectal neoplasm; 38% of all detected adenomas were flat lesions. This prevalence was similar to that reported by Rembacken *et al*. (2000) but was higher than that reported by Saito *et al*. (2001) and Wolber and Owen (1991) in the USA and Canada, respectively. It has also been shown that these lesions have a predilection for the development of high-grade dysplasia and early invasive carcinomatous change. Lesions with these characteristics tend to be found predominantly in the right hemicolon. The genetic mutations responsible for this *de novo* morphological model are still unclear but there have been insights into their clinicopathological importance that are described later in this chapter.

ENDOSCOPIC OPTICAL BIOPSY TECHNIQUES

Colonoscopy was first introduced as a means of directly visualizing the colon in the 1960s. Apart from its therapeutic adjuncts, there have been advancements in image acquisition during colonoscopy. High magnification chromoscopic colonoscopy (HMCC) allows magnification of the mucosa up to 150 times and enhanced digital imaging improves image resolution. Commonly used dyes include indigo carmine (non-reactive, non-absorbable), methylene blue (reactive, absorbable) and crystal violet (absorbable and potentially toxic). The delineation of lesions of all kinds was markedly improved with this technique, and subtle lesions can be identified by seeking the following mucosal signs (Figures 10.1A, B):

- Focal pallor or erythema
- Haemorrhagic spots
- Fold convergence
- Disruption of mucosal vascular net pattern
- Mucosal unevenness or discrete mucosal deformity
- Air-induced deformation

Once identified, the suspicious areas are washed down with the appropriate mucosal toilet and the dye applied. Indigo-carmine and methylene blue require saline mucosal toilet, but mucolysis with a proteolytic compound (e.g., acetylcysteine) is required prior to crystal violet application. Lesions are then sized using an open biopsy forceps whose width is known or by using an

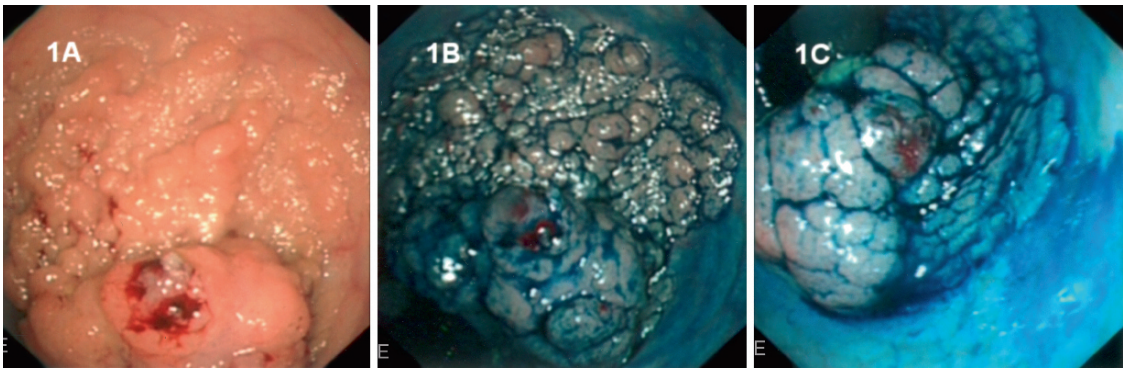

FIGURE 10.1 **(A).** Conventional white light colonoscopic views of a distal sigmoid lateral spreading tumour (G-LST). **(B)** Indigo carmine 0.5% chromoscopy has been applied to the mucosa delineating the lateral margins of the lesion. **(C)** Retroflexion views of the lesions shows retrofold disease, not seen using *en face* views

Table 10.1. Schematic summary of the Paris morphological classification system of colorectal lesions.

Endoscopic appearance	Paris class		Description
Protruded lesions	Ip		Pedunculated polyps
	Ips		Subpedunculated polyps
	Is		Sessile polyps
Flat elevated lesions	0-IIa		Flat elevation of mucosa
	0-IIa /c		Flat elevation with central depression
Flat lesions	0-IIb		Flat mucosal change
	0-IIc		Mucosal depression
	0-IIc / IIa		Mucosal depression with raised edge

'endo-rule'. Morphological classification using the Paris Workshop classification guidelines (2002) (Table 10.1) is undertaken, and then more details are obtained by activating the colonoscope's magnification lever and characterizing lesions' appearances.

The structure of the colonic pits is also visually enhanced by the dye and the resulting patterns can be characterized (Figures 10.2A, B). Pit patterns have been shown to correlate strongly with their associated histopathological diagnoses and as a result, this method of classification has become popular. Types I and II are associated with normal and hyperplastic mucosa, respectively. Type IIIs are seen more often in depressed lesions and are associated with carcinomatous change. Type IIIL are associated with adenomas in protuberant lesions. Type IV are associated with adenomas with atypical cellularity whereas type V or non-pit patterns are indicative of adenocarcinoma (Table 10.2). The use of crystal violet is usually limited to the identification of patterns with Type V characteristics.

Various studies have investigated the usefulness of HMCC as a means of providing an optical biopsy of protuberant and flat lesions. In an analysis of 1,008 flat and depressed lesions, the Sheffield

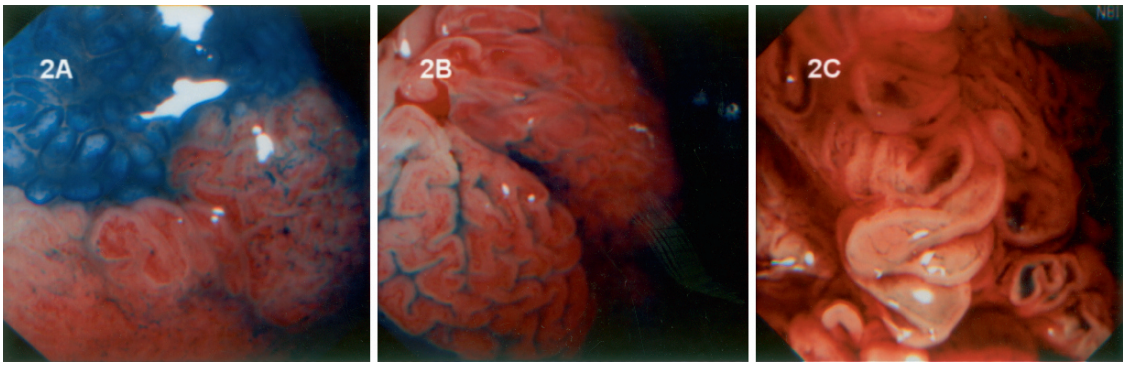

FIGURE 10.2 **(A).** high-magnification chromoscopic views (100x magnification) shows a neoplastic type IV crypt pattern adjacent to a 'villiform' background mucosa at the site of previous attempted EMR. **(B, C)** High-magnification views (100x magnification) of the central protruding nodule using indigo carmine 0.5% chromoscopy and narrow band imaging. A neoplastic type IV crypt architecture is seen. There is no evidence of an invasive type V crypt to infer deep submucosal invasion

TABLE 10.2. The modified Kudo criteria for the classification of colorectal crypt architecture *in vivo* using high magnification chromoscopic colonoscopy.

PIT TYPE	CHARACTERISTICS	APPEARANCE USING HMCC	PIT SIZE (mm)
I	Normal round pits		0.07+/– 0.02mm
II	Stella or papillary		0.09 +/– 0.02mm
IIIs	Tubular / round pits Smaller than pit type I		0.03 +/– 0.01mm
IIIL	Tubular / large		0.22+/– 0.09mm
IV	Sulcus / gyrus		0.93 +/– 0.32mm
V(a)	Irregular arrangement and sizes of IIIL, IIIs, IV type pit		N/A

group in 2004 showed the association between pit patterns and histopathology. When subgrouped into neoplastic (IIIL, IV, IIIs, V) and non-neoplastic (I, II) classes, the sensitivity of HMCC and pit pattern analysis at distinguishing neoplastic from non-neoplastic lesions was 98%, with a positive predictive value of 95%. Kato *et al*. (2006) had an overall diagnostic accuracy of 99.1% in an analysis of 210 morphologically unspecified lesions. The diagnostic accuracy for non-neoplastic pit patterns (negative predictive value) was 100% (24/24). The accuracy of neoplastic pit patterns (positive predictive value) was 99.8% (184/186). Tung *et al*. (2001) showed that the sensitivity and specificity of diagnosing neoplasia from flat and protuberant lesions were 93.8% and 64.6%, respectively. Despite these encouraging results, the routine implementation of HMCC enhanced pit pattern characterization is hindered by these variations in overall accuracy, sensitivity, and specificity in differentiating between neoplastic and non-neoplastic lesions (Kato *et al*., 2006). This discrepancy may be caused by differences in operator experience, chromoscopic technique and histological interpretation. There are limited published data reporting on the learning curve required to fulfill the primary end points of competence and sustained observer accuracy and inter/intra observer variability. It is clear from the above studies, however, that there is an increase in overall accuracy rates with more experience of the procedure and of pit pattern analysis.

High magnification chromoscopic colonoscopy with Paris classification and Kudo pit pattern characterisation has been shown to be a useful tool for surveillance in patients with chronic ulcerative colitis. These patients are known to form morphologically heterogeneous lesions that have traditionally required careful surveillance with an intensive biopsy load. The terms 'DALM' (dysplasia-associated lesion mass) and ALM (adenoma like mass) attempt to differentiate between these lesions. The management and clinical interpretation of dysplasia in the context of chronic ulcerative colitis is radically different from that of sporadic dysplastic lesions in the 'normal' population. Data from two other groups confirm these data. Presently, dysplasia is the most reliable biomarker of malignant change, being present in > 70% of ulcerative colitis patients with CRC. Dysplastic lesions require colectomy, whereas adenomas are subject to intensive surveillance. Recent published data suggest that using HMCC with targeted biopsies in the context of CUC surveillance significantly increases fourfold the diagnostic yield for intraepithelial neoplasia (IN) as opposed to conventional colonoscopy and biopsy protocols. The ability to differentiate IN from hyperplastic or inflammatory mucosal change using HMCC in ulcerative colitis has also been shown to offer a sensitive and specific tool with a high overall diagnostic accuracy (96%) (Hurlstone *et al*., 2004b, 2005).

ENDOLUMINAL STAGING OF COLORECTAL LESIONS

The correlation between pit pattern analysis and tumour staging in Paris class 0-II early colorectal cancers is important. This is because those limited to submucosal layer 2 can be managed by endoscopic mucosal resection as the risk of recurrence, lymphovenous invasion, or lymph node

metastases is < 8% (Kikuchi *et al*., 1995). Deeper lesions are associated with a much higher risk of lymph node metastases, non-curative resection, procedural complications (perforation), and require surgery. Saitoh *et al*. (1998) used the following criteria to determine submucosal layer 2 invasion with an overall accuracy of 91% in 64 lesions (specificity 70%): presence of expansion appearance; deep surface depression; uneven irregularity of depressed surface; and converging folds toward the tumor. The area and diameter of the lesion covered by Kudo type V pit pattern has been shown to correlate closely with the degree of submucosal invasion in sessile, flat, or depressed carcinomas. Tanaka *et al*. (2002) reported that lesions with a maximum pattern area diameter of > 5 mm showed a depth of submucosal invasion of > 1,500 μm. This study used post-resection stereomicroscopy to determine these characteristics but the authors suggested that magnification colonoscopy can be used to estimate the pattern area and hence direct therapeutic decisions. Furthermore, Nagata *et al*. (2000) demonstrated that the characterization of subtypes of the Kudo type V pit pattern may also be indicative of the degree of submucosal invasion. The implementation of Nagata subtype analysis of the Kudo type V pit pattern in this study showed a 0.51 κ coefficient of agreement between pit type V and histologically confirmed sm2 invasion. Using pit types Vn(B) and Vn(C) as clinical indicators of invasive disease, 97% of lesions were correctly anticipated to have sm2+ invasion, with low specificity (50%, overall accuracy 78%), implying a tendency to overstage lesions using this analytical technique.

Narrow-band imaging (NBI) is a promising new development in colonoscopic imaging. Instead of using conventional white light to illuminate the mucosa, the light passes through R/G/B filters on its path from the source. The depths of penetration of the light from these spectra differ within the mucosa and therefore, with the push of a button different mucosal layers can be selectively visually enhanced. It has the advantage of not only enhancing the mucosal characteristics of colonic lesions but also enhancing their vascular architecture (Figure 10.2C). It also does not require the occasionally time-consuming process of dye spraying. Pit pattern analysis and the differentiation of neoplastic and non-neoplastic lesions with NBI may be comparable to that with HMCC, but little work has been done on tumor staging using narrow-band imaging characterization.

Recently, the incorporation of a laser scanning microscope to a conventional video colonoscope has allowed the *in vivo* detection of neoplasia as well as estimation of depth invasion. Laser scanning confocal microscopy (LCM) is an adaptation of light microscopy whereby focal laser illumination is combined with 'pinhole limited detection' to geometrically reject out of focus light. This technology allows visualization of the mucosa and submucosa to a depth of 250 μm below the mucosal surface. Optimized views are obtained with the use of fluorescein intravenously which is taken up by the cell to provide high contrast image but, acriflavine, tetracycline or cresyl violet can be used topically on the mucosa as image enhancers. This novel method is currently being validated against conventional histopathology in Western centres and, as of yet, has not been used as a staging tool.

In addition to using image enhancing techniques, polyp morphology and pit

pattern analysis to stage colorectal lesions, *in vivo* real-time ultrasonography can be used. Conventional 7.5 MHz endoscopic ultrasonography (EUS) is most commonly used for staging rectal tumours. However, these instruments are large, rigid, and cannot be introduced via colonoscope. For this reason, amenable lesions identified by colonoscopy require the reinsertion of the transducer and reidentification of the lesion, which is time consuming and inconvenient for the patient. Mini-probe technology has allowed the development of high frequency (12.5–20 MHz) ultrasound probes that can be introduced through the side port of a conventional colonoscope. Acoustic coupling is achieved by water immersion of the colonic segment being imaged or by inflating a small balloon sheath at the end of the transducer with water (Tseng *et al*., 2002). The mini-probe is advanced to the proximal end of the lesion and gradually withdrawn distally providing a real-time 360° radial image. Extramural imaging is sensitive enough to identify associated lymph node metastases. A large prospective study was performed by Hurlstone *et al*. (2005) in which 131 colonic tumours underwent high-frequency EUS analysis followed by either endoscopic mucosal resection, or surgical resection and subsequent histopathological analysis. T staging was possible in 99% of lesions. Two lesions (1.5%) were over-staged and three (2.3%) were under-staged with T0 (adenoma) and T1 lesions undergoing EMR. Nodal staging was less accurate with 87% of lesions correctly N staged, 3/93 (3.2%) lesions under-staged and 9/93 (9.7%) over-staged. Similar studies (e.g., Tseng *et al*., 2002) have shown high accuracy rates (82–90%) for T staging, but N staging was less accurate (73–87%). The heterogeneity of these results may be influenced by operator experience, differing penetration by higher frequency transducers, differences in acoustic coupling and the routine use of a single channel colonoscope. The use of ultrasound in the staging of invasive colorectal cancers and the staging criteria, investigated by Cho *et al*. (1993), are summarized below:

- uT0: lesions confined to the first hypoechoic layer
- uT1: lesion penetration to the third hypoechoic layer
- uT2/3: lesion penetration of the muscularis propria (outer hypoechoic layer) or serosa (most peripheral echogenic band)
- uT4: lesion extension through the serosa with or without infiltration of adjacent structures
- u node positive (N+): presence of pericolic, well demarcated hypoechoic round or oval structures > 10 mm diameter

ENDOSCOPIC MUCOSAL RESECTION: RATIONALE AND METHODOLOGY

The aim of these techniques is to facilitate the identification of patients with severe dysplasia and T1 disease without SM3 invasion, Saitoh's invasive characteristics or an invasive Kudo type V pit pattern. As mentioned earlier, any lesion with these characteristics is associated with an increase in lymphatic invasion and lymph node metastases, making surgery the only option for curative resection. In these carefully selected patients, curative resection is possible by endoscopic mucosal resection. This is a minimally invasive technique

that obviates the need for open surgery; hence, minimizing post-operative pain, enhancing the post-operative return of gut motility, reducing hospital stay and preventing the comorbidity associated with open surgery. Given Moreaux's (1989) long-term survival and prognosis data for early CRC (5-year survival > 90%) endoscopic mucosal resection may represent an alternative therapeutic option from both a patient preference and health-economic perspective, but further studies comparing conventional surgery to EMR with appropriate cost effective modeling are required.

Endoscopic mucosal resection was originally described by Dehyle *et al*. (1973), and has been developed by Japanese endoscopists for the resection of sessile and flat lesions of the stomach, esophagus, and colorectum. Simple snare resection is sufficient for pedunculated lesions. EMR permits the resection of flat and sessile lesions by longitudinal section through the submucosal layer. EMR facilitates complete histological analysis of the resected lesion and makes it possible to determine precisely the completeness of excision in both the horizontal and vertical resection planes. This makes it advantageous compared to primary tissue ablative techniques such as argon plasma coagulation and electrocoagulation.

The technique of EMR comprises four stages:

1. Diagnosis and localisation of the lesion
2. Evaluation of invasive depth to exclude lesions invading the deep submucosal layer 3 or beyond (i.e., T2 disease) using HMCC or ultrasound techniques
3. Excision procedure
4. Post resection evaluation

Initial diagnosis and location of flat and sessile lesion of the colorectum is facilitated by the use of HMCC and allows the observation of detailed morphology. *In vivo* staging of identified lesions is then performed using morphological and pit pattern analysis or through-the-scope mini-probe ultrasound technique. Flat and sessile lesions up to 20 mm in diameter can be resected by *en bloc* or 'single pass' resection with larger lesions requiring a piecemeal approach. A needle catheter is then inserted through the side port of the colonoscope with sterile saline injected around the lesion and surrounding mucosa. A cleavage of the submucosa (having the effect of raising the lesion) then permits simple snare resection. A single cannulation can be used for small lesions of < 10 mm diameter with multiple cannulations usually required for lesions of 20 mm or larger. Some authors advocate the use of adrenaline (1/100,000) mixed with saline or the use of twice-normal saline at submucosal injection. There are no randomised controlled trials proving the superiority of these methods with regard to resection clearance, post EMR hemorrhage or perforation. Whatever injection medium used, it is essential to maintain a sufficient mucosal lift or detachment throughout the EMR, which minimises the risk of muscularis propria entrapment and subsequent perforation. Some lesions are unsuitable for conventional EMR despite favourable T and N staging. These are lesions that are anatomically inaccessible by colonoscope (i.e., behind folds) or lesions that spread over two consecutive folds or occupy more than a third of the luminal circumference (due to the high risk of stenosis).

We advocate peripheral margin tattoos or thermal mucosal cautery marking prior

to saline submucosal injection to delineate the normal mucosal boundaries around the lesion prior to snaring. This is a helpful technique, as at submucosal lift, the lesion can become distorted and indistinct from the surrounding normal mucosa. If the lesion fails to lift (the non-lifting sign of Uno) or has an asymmetrical appearance, then the resection should be abandoned as this indicates tethering to the underlying muscularis mucosa. Perforation and risk of non-curative resection can occur in this scenario.

Following successful submucosal lift, a spiked or 'barbed' snare is applied over the lesion and slowly closed under gentle suction. This permits the lesion to be retained within the snare boundaries before final resection. Prior to final cutting (usually using a 25 W coagulation current) the snare should be relaxed slightly to allow any entrapped muscularis mucosa to retract. Following resection, the lesion is retrieved using a pronged grasping forcep or Roth net, followed by immediate fixation in 10% formalin solution. Japanese endoscopists 'pin out' the lesion onto a solid cork or polystyrene plate prior to fixation that limits shrinkage of the resection specimen and permits easier and more accurate histopathological sectioning.

In summary, the exclusion criteria for EMR are:

- Lesions amenable to simple snare polypectomy (Paris type Ip, Isp lesions)
- Lesions demonstrating Kudo IIIs/V pit patterns that suggest deep submucosal invasion
- Lesions showing asymmetric lifting on submucosal injection
- Lesions more than 20 mm in diameter
- Uncorrected coagulopathy

ENDOSCOPIC SUBMUCOSAL DISSECTION: RATIONALE AND METHODOLOGY

Following resection, it is important to reevaluate the cut margin of the mucosa to ensure that no neoplastic tissue is left *in situ* as this continues to assume a risk for carcinomatous change. High rates of adenoma recurrence, despite reported complete excision by the endoscopist, have been reported. HMCC has been shown to provide a sensitive and specific tool to help guide the endoscopist as to whether resection was complete or not. The Sheffield group demonstrated that for both *en bloc* and piecemeal resections, HMCC as an *in vivo* tool to predict remnant tissue post EMR had an overall accuracy of 95% (Hurlstone *et al*., 2004a). This enables the identification of patients at risk of incomplete resection, and hence recurrent disease, who require immediate extended EMR, more intensive endoscopic follow-up or alternative management strategies. Should a further EMR be unsuccessful, argon plasma coagulation (APC) of any remnant tissue, including application to the entire circumference of the cut margin can be applied. All lesions should have an adjacent submucosal tattoo applied using Indian ink to facilitate localization at follow-up colonoscopy.

Piecemeal resection is obviously not ideal as the default staging of the resection becomes Rx as opposed to R0 with adequate *en bloc* resection. In a recent analysis of 58 lateral spreading tumours (> 10 mm in diameter with a low vertical axis extending laterally along the luminal wall) 36 lesions required piecemeal resection due to their maximum diameter exceeding 20 mm, and the majority of recurrences

(8/10) detected occurred in this group (Hurlstone *et al*., 2004c). These recurrences were successfully managed by further EMR. The problem of recurrence that piecemeal or incomplete resection poses may be tackled by utilizing endoscopic submucosal resection (ESD) which has recently been developed by Japanese groups for the endoluminal resection of Paris 0-II lesions of the stomach, gastro-esophageal junction and esophagus using a gastroscope with a distal transparent cap attachment. The technique allows *en bloc* knife dissection after sodium hyaluronic acid or glycerol submucosal infiltration for lesions > 20 mm in diameter.

As with EMR, the ESD procedure begins with the characterization and endoscopic staging of the lesion. Following morphological classification using the Paris classification and modified Kudo criteria, Cho criteria are used to differentiate tumor stage and nodal disease status using high frequency ultrasonography. The exclusion criteria for ESD are:

- T2/N1 disease
- Evidence of hepatic/local nodal metastasis at index trans-abdominal computerized tomographic imaging (CT)
- Fixed type IIc component (defined as per Kudo criteria as constant concavity whether air is insufflated or deflated)

Cleavage of the submucosal plane from the muscularis is achieved using a 1% solution of 1,900 kDa sodium hyaluronic acid (HA) (19 ml) diluted with 1 ml of 1/10,000 adrenaline and 10 ml of 0.5% IC to facilitate identification of the muscularis dissection plane. Submucosal catheterization is accomplished a 23G disposable needle catheter, with working in a proximal to distal plane allowing 3–4 mm of 'normal' mucosal clearance throughout the horizontal axis of the lesion. No upper volume limit is set, with the primary end point of submucosal catheterization being a sustained and complete submucosal lift sufficient for knife dissection.

Following submucosal catheterization and 'lifting', circumferential mucosal incisions were made at 5–6 mm intervals around the pan-circumference of the lesion using a 'flex knife' fixed at a total cutting vertical length of 1 mm using a 40 W pure cut current. Submucosal dissection was then initiated from the most proximal lesions aspect using a 40 W cut current using the insulation tipped (IT) knife in an oblique 30–40° axial position (Figure 10.3). Partially resected

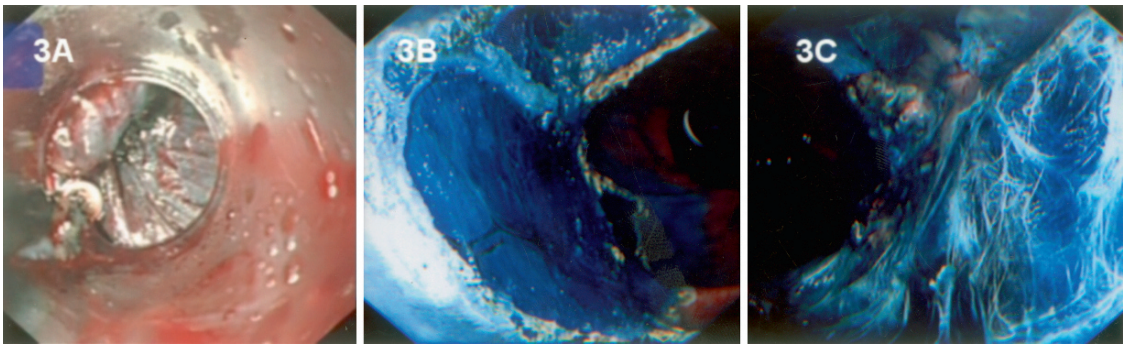

FIGURE 10.3 **(A).** The distally fitted transparent cap is seen with the distal tip of the insulation tipped needle knife at the 7'o clock position. The dense submucosal fibrosis (see as white strands) are being divided from the underlying muscularis propria. **(B)** Retroflexion views of the final endoscopic submucosal dissection plane. Note the blue standing of the underlying muscularis. **(C)** En face views of the final dissection plane

tissue is manipulated outside the dissection plane (required to permit continuous visualization of the dissection axis) using patient position change (i.e., combination right/left lateral and supine) in addition to direct tissue traction from the distally attached endoscopic dissection assistance cap. Mucosal defects need not be routinely closed using endoclips. Focal bleeding can be controlled by endoclip application and small capillary ooze by using argon plasma coagulation (APC).

RETROFLEXION ENDOSCOPIC DISSECTION

Endoscopic resection techniques have been described above as an *en face* technique i.e., distal to proximal forward viewing with the endoscope. This technique may be limited by proximal spread of neoplasia behind colonic haustrae necessitating retroflexion of the endoscope in order to obtain these views (Figure 10.1C). Currently, only conventional gastroscopes have a retroflexed width small enough to allow visualization and therapeutic strategies within the colon, but their use may be associated with reduced caecal intubation rates.

Lesions are initially examined using forward view and full retroflexion to characterise the most proximal, distal and luminal circumferential spread (Figures 10.3B and 10.4). Retroflexion views are then obtained by insertion of the endoscope tip 2–3 haustral folds proximal to most distal aspect of the lesion visible *en face* using 180° of anti-clockwise torque and maximal wheel angulation. Using targeted indigo-carmine dye spraying, lesions are then sized, morphologically classified using Paris Workshop criteria and staged

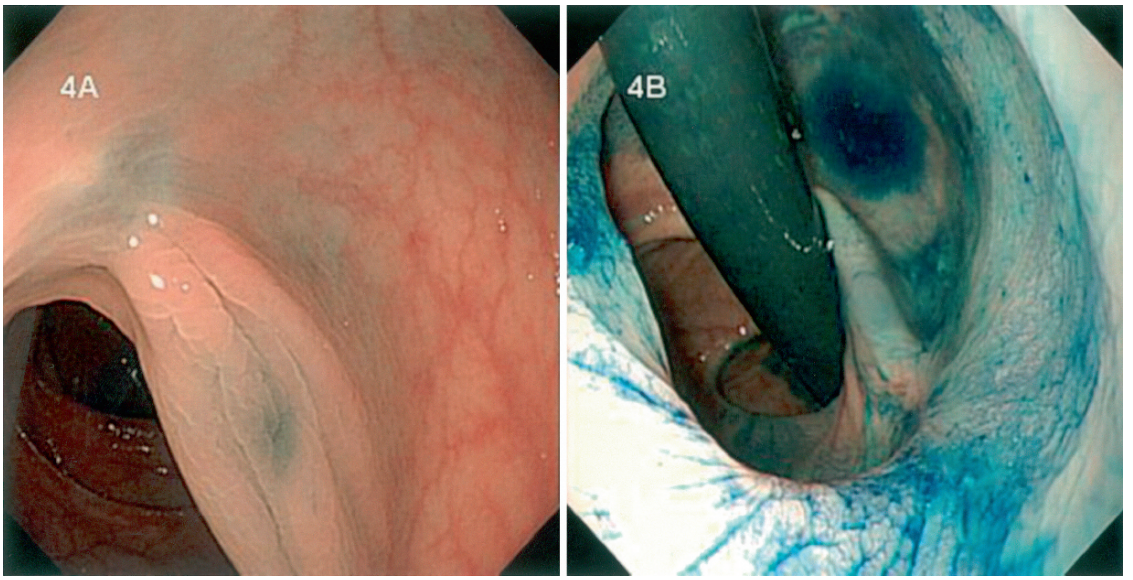

FIGURE 10.4 **(A).** En face views of the distal sigmoid at 6 months post dissection. The previously placed tattoo can be seen with fold convergence compatible with previous dissection. There is no endoscopic evidence of locally recurrent or residual disease. **(B)** Post dissection retroflexion views of the lesions at 6 months post dissection. Fold convergence is present but with no evidence of residual disease using 0.5% indigo carmine chromoscopy

using high frequency ultrasonography. Depending on the size, accessibility and morphology of the lesions, EMR or ESD can be performed as described earlier. The procedures are more technically complex in the retroflexed position, however, because of the counterintuitive movements required during instrumentation.

The Sheffield group published a series of 68 lesions referred for retroflexion EMR or ESD; 11% of the surveillance cohort required a second EMR due to recurrence at 3 month colonoscopic follow-up but had no further recurrence up to 24 month follow up. One caecal lesion was shown to have recurred at 12 months follow up and the patient was referred for surgery, the histopathological diagnosis being T1 disease. The overall 'cure' rate at 24 months follow-up was 98%. The bleeding complication rate in this series was 7% (all immediate or procedural) none of which required transfusion or surgical intervention. Three patients suffered postresection ileus but there was no case of luminal perforation reported (Hurlstone *et al*., 2006).

ENDOSCOPIC RESECTION IN CHRONIC ULCERATIVE COLITIS

Endoluminal resection techniques are not directly transferable to patients with chronic ulcerative colitis (CUC) because of the difficulties in differentiating between dysplastic and non-dysplastic lesions. Dysplastic lesions are traditionally referred directly for colectomy, but Rubin *et al*. (1999) and Engelsgerd *et al*. (1999) have hypothesised that because adenomas in CUC assume a risk of dysplastic and cancerous change in the order of 4% over a period of about 7 years, they can be managed by surveillance alone. Hurlstone *et al*. (2007) compared EMR in patients with CUC with EMR in matched controls with a moderate-high lifetime risk of colorectal cancer. They showed that, with the application of Paris morphological classification and modified Kudo criteria to colonic adenomas, EMR can be safely achieved in these patients with no difference in procedural or oncological outcome, and proposed that EMR can be added to the armamentarium in the treatment of dysplastic lesions in CUC.

POST RESECTION PROTOCOLS

In the immediate post-procedural period, patients should be observed for signs of colonic perforation and bleeding after EMR or ESD. This usually entails an overnight stay to ensure the absence of persistent abdominal pain with haemodynamic and temperature monitoring. The absence of long-term data regarding these advanced techniques necessitates intensive postresection colonoscopic surveillance. Recurrent neoplastic disease is defined as tumor present at the previous resection site, tumor evident with associated fold convergence or tumor (in the absence of fold convergence) 1–2 mm adjacent to the EMR mucosal scar (Higaki *et al*., 2003). Patients with signs of recurrence require reevaluation of the lesion using HMCC and modified Kudo pit pattern analysis. EMR/ESD can then be applied unless there are signs of invasive disease (see exclusion criteria described earlier) when surgery is advocated.

SAFETY AND APPLICABILITY OF ENDOSCOPIC RESECTION TECHNIQUES

The main complications of endoscopic resection are hemorrhage, perforation and stenosis. The immediate and early complications (10% of cases) described in the first 12 h post resection are principally hemorrhage and rarely perforation. Japanese and UK investigators have reported hemorrhage rates post colonic EMR of ≥ 2% in large cohorts. Okamoto *et al*.'s (1996) review of interventional colonoscopic resections also reports a perforation rate secondary to EMR of only 0.35% with Kaneko *et al*.'s (1995) multi-centre analysis showing mortality rates < 0.0001% for this procedure. EMR may therefore be a safe and effective endoscopic therapy that can enhance our current strategies aimed at the secondary prevention of colorectal cancer. The studies outlined earlier have now validated the use of these resection techniques in the West and may change the management of early colorectal neoplasia away from that of primary surgical resection, to that of endoscopic based resection, a procedure requiring a short hospital stay with minimal associated morbidity and mortality when compared to primary surgical resection.

Accurate *in vivo* staging is essential at colonoscopy prior to consideration of local endoluminal resection. Flat focal submucosal invasive CRCs which are limited to the submucosal layer 1 can be managed by EMR as the risk of lympho-venous invasion and nodal metastasis is rare (< 5%). For lesions with deeper vertical invasion into the submucosal layer 3 or beyond (stage T2), the risk of nodal disease increases to 10–15%. EMR in this group is, therefore, undesirable due to a higher risk of perforation, non-curative excision and untreated nodal disease. Surgical excision is recommended in this group.

The advantages of EMR are only applicable if early CRC is reliably and appropriately diagnosed and treated by an endoscopist who has received the appropriate specialist endoscopic training and maintained an adequate skill level. Currently, it would appear that Western endoscopists with conventional endoscopic training are less successful than Japanese endoscopists at diagnosing early stage cancer. This is not because the Japanese detect cancers by screening the complete asymptomatic population as even in Japan, most cases of early cancer are incidental findings at endoscopy. At the National Cancer Centre in Tokyo, 20% of colonic cancers are now diagnosed in the intramucosal or T1 stage and hence numerous data regarding the efficacy of EMR exists due to therapeutic demand. Importantly, increasing evidence suggests a similar prevalence and high grade dysplasia rate amongst flat and depressed lesions to Japanese authors, which thus emphasizes the importance of Western studies further validating endoscopic resection techniques outside of Japanese endoscopic practice.

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11
Role of Stromal Variables in Development and Progression of Colorectal Cancer

Xiao-Feng Sun and Hong Zhang

INTRODUCTION

Majority of cancer researchers have concentrated their efforts on tumor cells themselves to study tumor biology, morphology, and functions during tumor development and progression. However, tumor stromal variables, such as blood and lymph vessels, various stromal cells and proteins around tumor cells, have not drawn enough attention although they are at least equally important in tumor development, progression, and even tumor therapy. Tumor angiogenesis and lymphangiogenesis are the processes for formation of new blood or lymph vessels within and around tumor mass. Stromal cells consist of various cell types such as infiltrating immune cells, fibroblasts, and endothelial cells. Extracellular matrix (ECM) is a complex structural entity around the tumor cells, and often referred to connective tissue or ground substance. The ECM is composed of three major classes of structural proteins (collagen and elastin), specialized proteins (fibrillin, fibronectin and laminin) and proteoglycans (van den Hooff, 1988).

It is of importance to understand the role of stromal variables in tumor development and progression in order to design appropriate therapy against angiogenesis and other stromal proteinases. Based on the knowledge gained from this field, a number of anti-angiogenesis elements and matrix metalloproteinases (MMPs) inhibitors have been recently developed for clinical trials. Inactivation of stromal proteins inhibits angiogenesis, lymphangiogenesis, tumor growth, invasion, and metastasis. Consequently, this can stabilize and inhibit the tumor growth. In addition, stromal cells, compared to the tumor cells, are unlikely to develop drug resistance, although some stromal proteins are tumor-derived. In gerenal, most of the stromal proteins are the products of stromal cells. One of the problems with traditional chemotherapy and radiotherapy is that they indiscriminately affect both growing normal and tumor tissue. Therefore, a therapy targeted to the stromal will minimize the side-effects of anti-cancer therapy. Several characteristics of stromal variables make them to be attractive therapeutic targets.

In this chapter, we focus on clinicopathological aspects of tumor stromal variables, such as angiogenesis, lymphangiogenesis, inflammatory infiltration, and particularly interesting new cysteine-histidine rich proteins (PINCH) and stromelysin-3 (ST3) in colorectal cancer (CRC).

ANGIOGENESIS AND LYMPHANGIOGENESIS IN COLORECTAL CANCER

Normal tissues have efficient barrier to block endothelial cell migration and tumor invasion. However, the tissue barrier can be broken down by new-formed stromal that is usually loose and oedematous, so that endothelial and tumor cells can easily penetrate it. The process to form the new stromal is called stromatogenesis, which is a common response to the messages delivered by tumor cells and parallels with tumor progression (Sivridis *et al*., 2004).

When a tumor grows to a certain size (> 1–2 mm^3), tumor cells start to secret various growth factors to stimulate its own vascular formation for its further growth. This process is called tumor angiogenesis. Because the new-formed capillaries are poorly covered by pericytes compared to the mature capillary, the tumor cells can easily penetrate the immature capillaries and start blood metastasis. The formation of new lymph vessels is called lymphangiogenesis. Lymphatic endothelial cells have poorly-developed junctions with large gaps between the endothelial cells, and are discontinuous or even absent basement membranes (Björndahl, 2005). Lymphangiogenesis is a new field concerning both basic and clinical research until the recent discovery of lymphatic vessel specific markers for identifying lymphatic vessels by immunohistochemistry (Figure 11.1). The landmark in this field is that vascular endothelial growth factor (VEGF) family members have been found to play a critical role in the lymphangiogenesis.

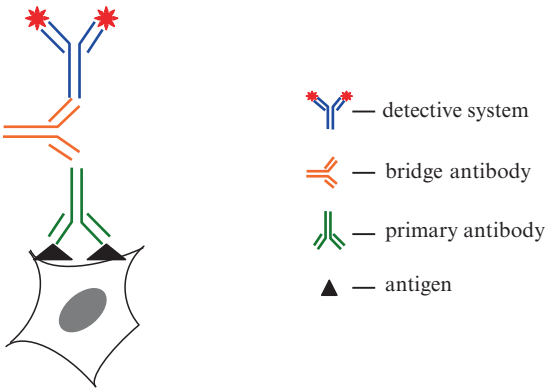

FIGURE 11.1. Principle of immunohisto(cyto)chemistry. A specific primary antibody recognizes and binds to the corresponding antigen in cell or tissue. Bridge antibody is then used to bind to the primary antibody. Finally, a detective system is added to visualize the presence of the antigen

Tumor cells together with leukocytes, macrophages, and mast cells secrete VEGF by both physiological and pathological stimulations such as platelet-derived growth factor (PDGF) and their receptors (PDGFR), insulin-like growth factor (IGF) and their receptors (IGFR), circulating endothelial precursor cell (CEPC), fibroblast growth factor (FGF)-2, angiopoietins, EphrinB2 and EphB4 (Björndahl, 2005). These factors, in turn, directly or indirectly are involved in angiogenesis and lymphangiogenesis in tumors. Among these factors, the VEGF family (VEGF-A, B, C and D) is the most characterized in angiogenesis and lymphangiogenesis by binding to various VEGF receptors (VEGFR): VEGFR-1 (also known as flt-1), VEGFR-2 (Flk-1 or KDR), and VEGFR-3 (Flt-4). VEGFR-1 organizes blood vessels with high affinity for VEGF-A and VEGF-B. VEGFR-2 binds to VEGF-A, VEGF-C, and VEGF-D and activates blood vessel proliferations. VEGFR-3 expressed in the lymphatic endothelial cells binds to

VEGF-C and -D, and plays a critical role in cell growth during lymphangiogenesis (Hanrahan *et al*., 2003).

MICROVESSELS IN TUMOR DEVELOPMENT AND PROGRESSION

Microvessel density (MVD) is associated with the expression of VEGF, especially VEGF-C found at tumor invasive site. Furthermore, MVD is gradually increased from normal mucosa to adenoma and carcinoma in the colon and rectum (Bossi *et al*., 1995; Fox *et al*., 1998), which is also found in the early stage of dysplasia, and gradually increases from low to high grades of dysplasia (Sharma *et al*., 2003). In tumors, MVD is increased when the tumor invades from the mucosa to the muscularis propria. However, the highest level of MVD has been found at the invasive margin of tumor, a site of active tumor invasion (Choi *et al*., 1998). Obviously, MVD is important in tumor development and progression. Furthermore, high MVD level is often found in CRCs with larger size (Onogawa *et al*., 2002), non-mucinous type, poorer differentiation, deeper invasion, lymphatic, and blood vessel invasion (Vermeulen *et al*., 1999), higher potential of recurrence and metastasis (Acikalin *et al*., 2005). Moreover, multivariate analyses show that the tumors with high levels of MVD have been correlated to advanced Dukes' stage (Koukourakis *et al*., 2005), and short survival in CRC (Li *et al*., 2003). Immature revascularization has been observed in poorly differentiated tumors, which is correlated with metastasis and poor prognosis. Thus, not only microvessel density but also vessel maturation is a crucial factor for tumor development and aggressiveness of CRC. On the other hand, high level of MVD has been found in the early stage of CRC (Shan *et al*., 2003), which correlates with longer survival for node-negative CRC patients (Khorana *et al*., 2003). Some primary tumors may produce various inhibitors to inhibit angiogenesis. Therefore, we sometimes find that when primary tumor is removed, release of the suppression results in the activation of angiogenesis leading to tumor metastasis (O'Reilly *et al*., 1994).

VASCULAR ENDOTHELIAL GROWTH FACTOR IN DEVELOPMENT AND PROGRESSION OF COLORECTAL CANCER

In general, VEGF (e. g., VEGF-A and -C) is highly expressed in early stages of colorectal adenomas, *in situ* carcinomas and invaded CRC as compared to their normal mucosa (Kuramochi *et al*., 2006). However, VEGF-D expression is decreased in both polyps and carcinomas than in the normal mucosa. The explanation for this is that the reduced VEGF-D allows VEGF-A and -C to bind to the VEGF receptors, and further switch on the angiogenesis for tumor growth. Clinically, VEGF has been associated with poor differentiation, deep tumor invasion, advanced Dukes' stages, and distant metastasis. Increased expression of VEGF, VEGF-C, and VEGF-D at the deep invasive site of tumors is correlated to poor prognosis (Akagi *et al*., 2000; Cascinu *et al*., 2000; White *et al*., 2002).

Moreover, VEGRR-2 or -3 is expressed in CRC and the normal colorectal mucosa. VEGFR-3 expression in the blood vessels is increased from normal mucosa to adenoma and carcinoma, which is associated with lymph node metastasis (Wang *et al*., 2005).

LYMPHATIC DENSITY IN STROMA OF COLORECTAL CANCER

There are only a few studies concerning the density of lymphatic vessels in stroma of CRC. A study in CRC shows that lymphangiogenic markers, such as LYVE-1, Prox-1, podoplanin, and 5'-nucleotidase, are highly expressed in cancer tissue rather than in the normal mucosa. Lymphatic vessels in stalk stromal are found to be associated with early tumor invasion (Fogt *et al*., 2004). Furthermore, lymphatic vessels have been recently reported to be present in majority of colon cancers (91%), and the density of lymphatic vessels is correlated with lymph node metastasis, but not with tumor size, invasion, and distant metastasis (Kuroyama *et al*., 2005).

Like blood vessels, newly-formatted lymph vessels in the center of tumors do not function as well as those at the invasive margin of tumors. Morphologically, intratumoural lymph vessels are often compressed and smaller, while those around tumors are often hyperplastic. These enlarged lymph vessels may function to collect tumor cells for further lymphatic metastasis. Moreover, VEGF-C and -D are associated with the density of large lymphatic vessels and metastasis to regional lymph nodes (Skobe *et al*., 2001; Stacker *et al*., 2001). Moreover, activation of angiogenesis, lymphangiogenesis, VEGF, and VEGFR is markedly increased in CRC compared to normal mucosa and adenoma, which is related to poor survival in the patients.

INFLAMMATORY INFILTRATION

Innate and adaptive immunities are the two types of immune responses. The innate immunity reacts rapidly with molecular patterns in microbes, independent of prior pathogen contact. Adaptive immunity is specific immunological memory and requires the pathogen recognized by the innate immunity. Immune responses play a critical role against tumors based on the recognition and specific binding to the surface components of the tumor cells and further killing of the tumor. Tumor inflammatory infiltration (TII) includes T, B, natural killer (NK) cells and macrophages. Anti-tumor effects of CD4+ and CD8+ T lymph cells are mediated by cytokine secretion (Fossum *et al*., 1994). B lymph cells from lymph node migrate to the tumors where they undergo antigen-driven proliferation to produce antibodies. The antibodies bind to the tumor cells and further destruct the tumor cells via phagocytes. NK cells are the lymphocytes that kill mutant and infected cells by granzyme release, such as perforins and chemokines, which activate the enzymes and lead to apoptosis of the mutant and infected cells by means of destruction of their structural cytoskeleton proteins and by chromosomal degradation. Subsequently, these target cells are broken down to small fragments and then removed by phagocytosis. Moreover, macrophages can inhibit the

tumor growth by secreting lytic enzymes such as lysosomal enzymes and TNF-α into tumor cells, which can be activated by IFN-γ and macrophage activation factor (Kaattari *et al*., 1980).

The TII response has dual effects on tumor development and progression to kill tumor cells and favorable patient or to produce cytokines and growth factors to further stimulate tumor growth and migration (Dimitriadou and Koutsilieris, 1997).

LYMPHOCYTIC INFILTRATION AND PROGNOSIS IN COLORECTAL CANCER

Lymphocyte infiltration has been associated with good prognosis in CRC patients, even after adjustment of clinicopathological variables (Gao *et al*., 2005; Menon *et al*., 2004). In addition, extensive TII has been correlated with better differentiation of tumors, earlier stage, lower rates of recurrence, and distant metastasis. There are several explanations for association of the TII with better survival in cancer patients. The TII has been considered as a barrier against tumor penetration. Clear evidence shows that the tumors with extensive TII respond often better to current chemotherapy. Moreover, our recent study in rectal cancer reveals that young patients with more TII around tumor cells have better immunological response than the older patients (Knutsen *et al*., 2006).

Although TII in the inner part of tumors is not significantly related to clinicopathological variables including patient survival, abundant TII at the invasive margin of the tumors is indeed correlated with a better prognosis in CRC patients, indicating that TII at the invasive margin is important to protect against the tumor progression (Gao *et al*., 2005). Colorectal cancers with infiltrative growth pattern in the invasive margin represent a malignant phenotype and often predict poor prognosis compared to tumors with expansive growth pattern (Fujita *et al*., 2003). In addition, expression of PINCH or PRL at the invasive margin, not in the inner parts of the tumor, has been associated with a poor prognosis in CRC patients (Gao *et al*., 2004; Wallin *et al*., 2006). Therefore, the invasive margin is considered as a critical area for angiogenesis, lymphangiogenesis, tumor invasion, and metastasis. There were six pathologists who observed 60 tissue slides from 30 colonic carcinomas, and five of them showed from good to excellent agreement concerning the invasive margin, without significant sampling error (Dundas *et al*., 1988). TII, especially at the invasive margin of tumor, indeed plays a critical role against tumor development and progression of CRC.

EXTRACELLULAR MATRIX AND MICROENVIRONMENT

Cell adhesion to ECM is mediated by the integrins. Focal adhesion (FA) is an integrin-rich cell adhesion site and contains cytoskeletal signaling molecules including FA kinase, integrin-linked kinase (ILK), talin, vinculin, and paxillin. ILK is an intracellular serine/threonine protein kinase regulating integrin-mediated cell adhesion, E-cadherin expression, pericellular fibronectin matrix assembly as well as cellular proliferation and survival. Cytoskeletal and signaling proteins are recruited to cell matrix contact sites where signals are transduced between the

intracellular signaling network ECM (Burridge and Chrzanowska-Wodnicka, 1996).

During tumor progression, tumor cells remodel the matrix either by expression or degradation of the ECM proteins to facilitate communication and escape control by the microenvironment. Furthermore, the remodeling of tumor microenvironment leads to releasing ECM-associated growth factors to suppress or stimulate tumor growth (Varani, 1987).

FUNCTIONS OF PINCH

PINCH at chromosome 2q12.2 encodes a 38 kDa protein with five LIM domains, which is first identified by a screening human cDNA library and later by Western blot (Figure 11.2) (Rearden, 1994), The PINCH is involved in protein–protein interactions, cellular proliferation, differentiation and survival. The LIM domains are the nuclear and cytoplasmic proteins and function as protein binding motifs with a cysteine-rich consensus sequence of ~ 50 amino acids folding into a three-dimensional structure with two zinc fingers, which are essential for embryonic development and tumourigenesis.

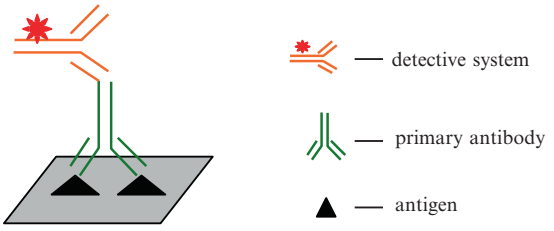

FIGURE 11.2. Principle of Western blot. A specific primary antibody recognizes and binds to the corresponding antigen on a membrane. Bridge antibody is then used to bind to the primary antibody. Finally, a detect system is added to visualize the presence of the antigen

PINCH interacts with the ILK through LIM1 domain binding to the first of four ankyrin (ANK) repeat domains at the ILK N-terminal. However, the C-terminal domain of ILK has certain homologies with the catalytic domains of serine/threonine protein kinases. This kinase-like domain interacts with cell-matrix contact sites such as CH-ILKBP (α-parvin, actopaxin), β1, β2, and β3 integrin cytoplasm tails, β-parvin (affixin) and paxillin. The PINCH, ILK, and CH-ILKBP form a ternary complex that interacts with other cell-ECM adhesion structures via multiple interactions. Overexpression of the N-terminus PINCH or ILK results in retarded cell spreading and reduced cell motility. Interactions of PINCH with ILK are crucial for regulation of cellular shape and migration. Inhibition of the formation of PINCH-ILK-CH-ILKBP complex leads to a significant reduction in fibronectin matrix deposition and reduction of cell proliferation (Guo and Wu, 2002; Zhang *et al*., 2004).

PINCH also binds to Nck2 through the LIM4 domain and SH3 domain 3 of Nck2, which, as an SH2/SH3 adaptor protein, is important in signaling pathways of epidermal growth factor (EGF) and PDGF receptors, and modulate actin dynamics by interacting with p21-activated kinase (Guo and Wu, 2002; Zhang *et al*., 2004).

Expression of PINCH in Different Organs

PINCH2 gene at chromosome 2q14.3 encodes a 39 kD protein that contains five LIM domains with 92% overall similarity to the PINCH1. In the embryo, PINCH1 is expressed in the heart, lung, kidney, liver, thymus, spleen, bladder, stomach, intestine, and skeletal muscle, while PINCH2 is restricted to the bladder, stomach, and intestine.

In the intestine, PINCH1 is expressed in epithelial cells and the smooth muscle layer, whereas PINCH2 is confined to the smooth muscle layer. In addition, PINCH1 is highly expressed in megakaryocytes during fetal liver hematopoiesis, but not the PINCH2. Megakaryocytes also express ILK and Nck2, the binding partners of PINCH1. In adults, both PINCH1 and PINCH2 are expressed in the heart, lung, kidney, liver, bladder, uterus, testis, skin, skeletal muscle, large intestine, and fat.

PINCH2 is also located in the ECM adhesion sites, but only LIM1 domain binds to ILK, suggesting that PINCH2 has potential to interact with other cell-ECM adhesion structures. In addition to regulation of the PINCH1-ILK interaction, cell spreading and migration, PINCH2 also participates in regulation of nuclear processes in the nucleus. However, PINCH2 does not bind to Rsu-1, a highly conserved leucine rich repeat protein and expressed in various mammalian cells, as PINCH1 does. Ectopic expression of the Rsu-1 inhibits anchorage-independent growth of Ras-transformed cells.

PINCH in Development and Progression of Colorectal Cancer

PINCH expression is further analyzed by immunohistochemistry with a polyclonal antibody in human tissues. PINCH is overexpressed in the stroma of breast, prostate, lung, skin, and colon cancers, compared to their corresponding normal tissues. PINCH has been found to be abundant in the stromal cells at the invasive margin of tumors. We have recently studied clinicopathological significance of PINCH expression in a large series of CRCs, and shown that the PINCH expression in the stroma is gradually increased from normal mucosa to primary tumor and to metastasis. Moreover, PINCH is strongly expressed at the invasive margin of primary tumors. The PINCH expression is associated with lymph node metastasis and predicts a poor prognosis independent of Dukes' stage, growth pattern, and grade of differentiation (Zhao *et al*., 2006). However, PINCH expression in the inner part of primary tumor is not correlated with patient survival and other clinicopathological variables, such as Dukes' stage, growth pattern, and grade of differentiation. Therefore, localization of the PINCH protein in tumors, especially at tumor invasive margin, is critical for its function in tumor development and progression.

PINCH protein has been also found in fibroblasts, myofibroblasts, and endothelial cells in tumor-associated stroma, indicating that the PINCH is indeed involved in tumor: stromal interactions, which may activate tumor cells and further tumor progression. Moreover, PINCH is strongly expressed in the endothelial cells of tumor vasculature, indicating that the PINCH protein is involved in tumor angiogenesis. Therefore, it is of interest to further investigate PINCH in cancer invasion and metastasis, tumor–stromal interactions, and even in prognosis.

FUNCTIONS OF MATRIX METALLOPROTEINASES

Matrix metalloproteinases in the ECM family secreted by both tumor and stromal cells play a major role in cellular differentiation, apoptosis, tumor angiogenesis, invasion, and metastasis. These proteinases can cleave interleukin-2 receptor

(IL-2R), an upregulator of T lymphocyte proliferation, and activate TGF, an important inhibitor of the T-lymphocyte response against tumors, thereby suppressing the activation of T lymphocytes. Application of a blocking peptide prevents the interaction of MMP2 with its substrates leading to reduced angiogenesis. When the tumor cells are introduced into MMP2 knock-out mice, the tumors developed in the knock-out mice show fewer amounts of blood vessels and slower growth compared to the tumors developed in the wild-type animals (Egeblad and Werb, 2002).

Matrix metalloproteinases are the products from different genes and classified by their functional and structural characteristics. Based on their sequence homology, the MMPs are further divided into collagenases, gelatinases, stromelysins, and matrilysins. The MMPs, unlike classical oncogenes, are not upregulated by gene amplification or mutations. Increase of MMP expression in tumors is due to transcriptional changes, resulting in activation of oncogenes or loss of tumor suppressors. For example, MMP7 is upregulated by a transcription factor PEA3, and MMP1 and MMP13 are downregulated by p53 (Sun *et al*., 2000). Moreover, the activation of MMPs is blocked by TIMP1 and TIMP2, the balance between MMPs and TIMPs is critical in controlling ECM turnover and maintaining matrix homeostasis. MMP11 (also called ST3) gene is initially identified at chromosome 22q11.2 in breast cancer (Basset *et al*., 1990). The term“stromlysin-3” is chosen because the protein has the same four-domain structure as the previously described stromlysins, and“stromlysin” correlated with ST3 RNA expression in stromal cells of breast cancer. According to the gene location, sequence of the putative ST3 catalytic domain, and functions, the ST3 belongs to a new MMP subfamily that differs from those reported by MMP genes on chromosomes 11, 16, and 1 (Basset *et al*., 1990), and contains a recognition site for convertase-like enzymes. The ST3 proenzyme, unlike most of other MMPs, is processed intracellularly and released as a mature enzyme. Like most other MMPs, which are activated outside the cells by other MMPs or serine proteinases, ST3 can also be activated inside the cells by intracellular furin-like serine proteinases.

STROMELYSIN-3 IN DEVELOPMENT AND PROGRESSION OF COLORECTAL CANCER

Stromelysin-3 is found in the basement membrane remodeling through releasing or activating growth factors or cytokines stored in the ECM. The ST3 degrades insulin-like growth factor-binding protein-1 (IGFBP-1), further leading to cellular proliferation and survival (Manes *et al*., 1997). Cancer cells when injected into *ST3*-null mice show an increased frequency of apoptosis and necrosis (Boulay *et al*., 2001) through releasing survival factors such as insulin-like growth factor (IGFs). Thus, ST3 plays an important role in cancer cell survival and tumor development.

Stromelysin-3 protein is expressed in fibroblasts in the stroma around tumor cells but not in the central part of tumors. Stromelysin-3 is undetectable with Northern blot, *in-situ* hybridization (Figure 11.3), or immunohistochemistry in the normal colorectal mucosa, lower in adenoma, and higher in primary CRC and metastasis in the lymph node and liver.

There is no significant difference in the ST3 expression between the primary and metastatic tumors as well as between inner part and invasive margin of primary CRC (Porte *et al*., 1995).

Stromelysin-3 levels are higher in the *de novo* group than in the ex-adenoma group. Histopathologically, the *de novo* group is expressed in the tumors with an infiltrative invasion pattern, indicating that ST3 has invasive potential in the CRC (Wlodarczyk *et al*., 2001). Furthermore, tumors with infiltrative growth pattern express higher levels of ST3 than those with expanding growth pattern (Skoglund *et al*., 2004). However, there is no difference in frequencies of ST3 expression between primary and metastatic tumors, suggesting that the ST3 is involved in the local invasion and early development of CRC, rather than in the late stage of CRC (Thewes *et al*., 1996).

In conclusion, cancer researchers have mainly focused on studying biological, morphological and functional alterations of cancer cells themselves. However, stromal variables, within or around the cancer cells, such as blood and lymph vessels, various stromal cells, proteins and peptides, have been neglected although it is also very important in the tumor development and progression. It has been recently reported that stromal–epithelial interactions influence cellular proliferation, differentiation, death, angiogenesis, motility, and genomic

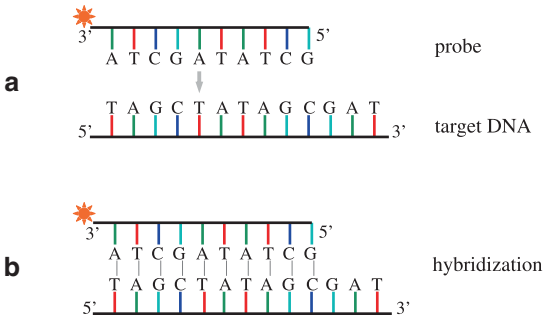

FIGURE 11.3. Principle of *in situ* hybridization of DNA. A specific probe labeled with a detective system binds to the complimentary DNA sequence in a cell (a), and visualizes the presence of the DNA sequence (b)

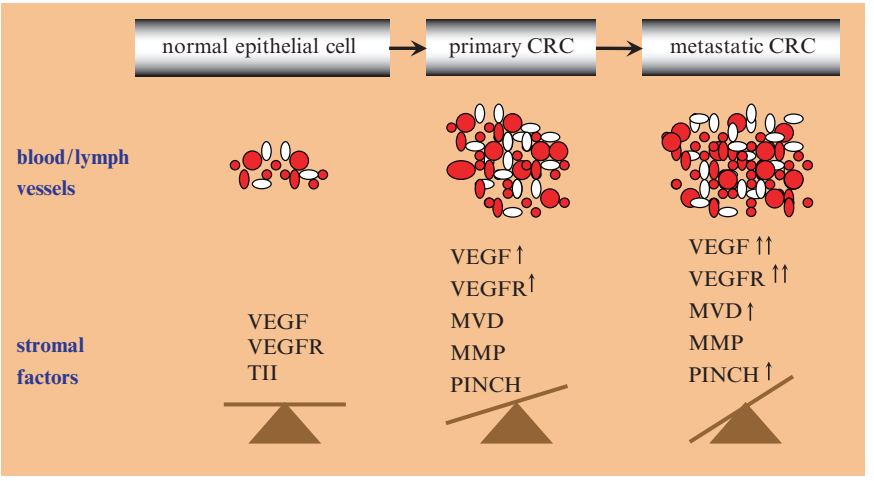

FIGURE 11.4. Alterations of tumor-associated stroma during development and progression of CRC from normal epithelial cell to primary CRC and further to metastatic CRC. Imbalance in the number and constructions of blood/lymph vessels, as well as the stromal factors promotes the process of tumor growth. (The illustration is modified from a review article by Sun and Zhang in *Mol. Cancer* 5: 43, 2006.)

integrity, and even cancer therapy. In this chapter, we mainly discuss clinicopathological significance of stromal variables such as angiogenesis, lymphangiogenesis, inflammatory infiltration, PINCH and ST3 in CRC. CRC is caused by both genetic predispositions and environments. The number of genetic alterations is increased from the first genetic change in a normal endothelial cell to several genetic alterations in the advanced stages of the cancers. In tumor stroma, the number and structure of blood and lymph vessels are altered by stimulations of many stromal factors, such as TII, VEGF, VEGFR, MVD, PINCH, and ST3. Interactions between these growth and anti-growth stromal factors in tumor-associated stroma affect tumor initiation, development, and progression (Figure 11.4).

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12
Quantitative Assessment of Colorectal Cancer Perfusion: Perfusion Computed Tomography and Dynamic Contrast Enhanced Magnetic Resonance Imaging

Vicky Goh

INTRODUCTION

Clinical interest in perfusion imaging for cancer has gained impetus in recent years due to developing clinical need and to technological advances that have facilitated such imaging. In oncology this has been driven by the development of drugs targeted at the tumor vasculature. Conventional assessment of the therapeutic efficacy of such anti-angiogenic and anti-vascular drugs has been shown to be of limited value in recent clinical trials. Such assessment is based on size change, e.g., Response Evaluation Criteria in Solid Tumours (RECIST) or World Health Organisation (WHO) criteria, yet these drugs may not necessarily cause tumor shrinkage. For example, a 5 month improvement in overall survival was reported in a Phase III study of patients with metastatic colorectal cancer, treated with conventional chemotherapy, and bevacizumab (Avastin; Genentech, San Francisco, CA, USA), a drug targeted against vascular endothelial growth factor; however, this was accompanied by an increase in objective response rate of only 10% (Hurwitz *et al*., 2004). While time-to-progression is probably the best method of assessing drug efficacy as it reflects disease stability, a disadvantage of using such progression as an endpoint in clinical trials is that large patient numbers may be needed, and such studies are expensive. Furthermore, patients could be treated potentially with ineffective drugs for prolonged periods. Perfusion imaging techniques such as perfusion computed tomography (CT) and dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) have been promoted particularly for early clinical studies (phase I and II) as these techniques may provide *in vivo* pharmacodynamic information. Such information may help in dose selection and scheduling, and in supporting decisions to take new therapeutic compounds forward to larger phase clinical studies with efficacy endpoints.

Both perfusion CT and DCE-MRI are attractive imaging techniques as they combine functional information regarding the tumor vasculature with good anatomical detail. Computed Tomography and MRI are widely available, and these perfusion techniques, which are based on contrast

media enhancement, can be incorporated relatively easily into standard imaging protocols. Both qualitative and quantitative information of tissue vascularity can be obtained. Using mathematical modelling to generate quantitative perfusion measurements, these techniques may demonstrate the increased vascular volume and flow within tumors, display the spatial and temporal heterogeneity of perfusion, demonstrate the hyperpermeability of the tumor vasculature, and provide a surrogate measure of tissue hypoxia. Of course, there are differences between techniques that should be taken into account. For example, a simple linear relationship exists between tissue enhancement and contrast concentration with CT, and quantification is relatively straightforward. In contrast, the signal intensity change with MRI is dependent on many factors, and not necessarily proportional to contrast dose, thus greater care has to be taken with quantitative perfusion assessment using DCE-MRI.

This chapter will overview current perfusion CT and DCE-MRI techniques for characterizing colorectal tumor vasculature, describe how quantitative data can be obtained with such techniques, and discuss the clinical utility of these techniques with particular focus on disease detection, disease characterization, prognostication, and therapeutic response.

TECHNICAL CONSIDERATIONS

Contrast Agents

Computed tomography contrast agents are typically iodine-based; MR contrast agents are largely gadolinium-based, although contrast agents with iron and dysprosium are available. The most commonly used contrast agents in clinical practice currently are low molecular weight agents, typically < 1 kDa, which diffuse freely between the intravascular and extravascular–extracellular compartments, but not across cell membranes into the intracellular compartment.

Contrast Kinetics

Perfusion CT and DCE-MRI are able to distinguish malignant from normal tissue by exploiting the differences in contrast agent kinetics between these tissues. As a freely diffusible contrast agent, bolus passes through a vascular bed, it remains confined to the intravascular compartment during the first pass of up to a few cardiac cycles. With the exception of some sites including brain, testis, and retina, contrast agent then diffuses from the intravascular into the extravascular–extracellular compartment at a rate determined by a number of factors including the rate of delivery, vessel surface area, and vessel leakiness or permeability. There is subsequent return of contrast from the extravascular compartment into the intravascular compartment over time, and contrast is eventually excreted by the kidneys, although some contrast agents have significant hepatic excretion.

In comparison with normal tissue, tumors typically show high permeability, and contrast will pass out into extravascular–extracellular compartment rapidly. For example, 12–45% of the contrast media may leak out in the first pass in breast tumors (Daldrup *et al*., 1998), thus contrast delivery may be the limiting factor of enhancement. Return of contrast into the intravascular compartment will also be faster, resulting in more rapid washout as plasma contrast medium concentrations

drop, in contrast with tissue showing low permeability such as areas of fibrosis and necrosis.

Contrast Administration

Contrast agents are administered typically as a bolus through a large bore cannula usually situated in an antecubital vein, with a saline chaser to optimize the contrast bolus. A pump injector suitably adapted for the imaging environment is used. Contrast dose and method of administration will vary depending on the imaging technique used and parameter measured. Doses in the order 0.5–1.5 ml/kg of 300 mg/ml or greater concentration of iodinated contrast are used for perfusion CT. Contrast doses in the order of 0.1–0.2 mmol/kg are used for DCE-MRI. T2* weighted sequences typically require a higher contrast dose than T1 weighted sequences. In general, the higher the contrast concentration, the greater the contrast volume used, and the faster the injection rate, the better the signal to noise ratio.

First pass studies provide information on blood flow and intravascular blood volume. Contrast is typically administered as a bolus injection for such studies. For perfusion CT, a typical bolus volume is 50 ml. The injection rate will vary depending on choice of mathematical modeling method. Lower injection rates of 3–5 ml/s are sufficient for deconvolution analysis, but higher rates of 5–7 ml/s are better suited for compartmental analysis. Typical bolus volumes for a first pass DCE-MRI study using a T2* sequence is 25–35 ml; this is usually administered at a rate of 5 ml/s.

Delayed phase studies to assess vascular permeability may be performed following contrast bolus injection, or following contrast infusion. Bolus volumes and rates for delayed phase perfusion CT are similar to that for a first pass study for assessment at a single tumor level. Infusional volumes of up to 100 ml at a rate of 2–4 ml/s can be used for volumetric perfusion CT. This optimizes the vascular and tissue contrast concentration for evaluation of permeability and intravascular blood volume using Patlak analysis, and has the advantage of permitting a large volume coverage, allowing whole tumors to be evaluated. For delayed phase DCE-MRI, a bolus volume of 10–15 ml, administered at a rate of 4 ml/s is typically used for a T1 sequence. This permits evaluation of the transfer constant (k_{trans}), which represents the diffusion rate into the extravascular–extracellular compartment, the extravascular–extracellular volume of distribution (V_e), and rate constant (k_{ep}), which represents the diffusion rate back into the intravascular compartment from the extravascular–extracellular compartment.

Contrast Concentration and Tissue Enhancement

The relationship between contrast concentration and enhancement is straightforward with CT; there is a direct linear relationship between enhancement change and iodine concentration. For example, at 120 kV, an enhancement change of 25 HU is equivalent to 1 mg/ml of iodine (Dawson, 1997), while at 80 kV, an enhancement change of 32 HU is equivalent to 1 mg/ml of iodine (Lee *et al*., 2003). As a result, the arterial input, required for quantitative analysis, can be measured directly from an artery included in the scan plane. Thus, absolute quantification of perfusion is possible using perfusion CT, and this has been hailed as the major advantage over DCE-MRI. In contrast, the relationship between

MR signal intensity change and contrast agent concentration is not so easily defined and indeed is nonlinear. Paramagnetic gadolinium based contrast media produce magnetic field inhomogeneities within the vascular space and in the immediate vicinity. This results in a decrease in relaxation time of the surrounding tissues. The MR signal intensity change is dependent on multiple factors including contrast medium dose, imaging sequence parameters, machine set up, and native relaxation rate of tissues. The arterial input required for quantitative analysis cannot be easily measured in the extracranial circulation due to artefact from velocity-induced signal intensity changes within vessels, and from the high contrast concentration within vessels. Quantification using pharmacokinetic modelling should only be performed if a direct relationship between signal intensity and contrast agent concentration can be demonstrated throughout the measured range.

Macromolecular Contrast Agents

Macromolecular contrast agents permit a more specific approach of assessing fractional vascular volume, and vessel permeability for two reasons. Firstly, unlike low molecular weight contrast agents that have a relatively high first pass extraction fraction for both normal tissue and tumor, macromolecular first pass extraction is low, of the order of $< 1\%$ for normal vasculature. As a higher proportion of intravascular contrast remains during the first pass, a more accurate vascular volume measurement is obtainable. Secondly, tumor microvessels are hyperpermeable to macromolecules in contrast to normally functioning vessels. This tumor vascular hyperpermeability allows plasma proteins to seep into the tumor interstitium, providing a favorable environment for subsequent ingrowth of tumor vessels (Gerlowski and Jain, 1986). This is a specific property of tumor neovasculature, which macromolecular contrast agents can assess.

To date there has been little data on macromolecular contrast enhanced perfusion CT. Imaging with PEG12000-Gen4-triiodo in experimental breast cancer in rats has shown that quantitative estimation of vascular permeability is possible (Simon *et al*., 2005). Similarly, a study in rats with chemically induced primary liver tumors has shown that early changes in hepatic flow can be demonstrated, and has the potential to allow tumor detection prior to development of overt lesions (Fournier *et al*., 2004). To date there has been no published data on macromolecular contrast enhanced perfusion CT in human subjects. Macromolecular DCE-MRI has been performed in animal tumor models to assess vascular permeability and vascular volume. Macromolecular MRI contrast agents based on gadolinium and iron oxide, varying from 5 to 90 kDa in molecular weight, have been investigated. The ability of such agents to characterize tumor vasculature is dependent on molecular weight, and physiochemical properties. Macromolecular extraction is slower than that for low molecular weight agents, and is related to molecular weight.

Several xenograft studies have validated albumin-$(Gd\text{-}DTPA)_{30}$ enhanced MRI for assessing tumor angiogenesis in a variety of tumor models, including mammary carcinoma, with a positive correlation

demonstrated between k_{trans} and vascular volume, and histological microvessel density (Van Dijke *et al*., 1996). Likewise, USPIO-enhanced MRI has been used to assess breast tumor permeability (Daldrup-Link *et al*., 2003) and good correlation between k_{trans} and tumor grade (Turetschek *et al*., 2001) and microvessel density (de Lussanet *et al*., 2003) has been reported. There have also been preclinical studies using albumin-$(Gd\text{-}DTPA)_{30}$ enhanced MRI, which have shown reductions in k_{trans} with anti-vascular cancer drugs (Turetschek *et al*., 2004). However, human use has been problematic. In addition to technical issues related to poor contrast to noise ratio, significant bone and liver retention of large sized molecules such as albumin-$(Gd\text{-}DTPA)_{30}$, and immunogenicity have been a concern.

DATA ACQUISITION AND MATHEMATICAL MODELING

Perfusion Computed Tomography

Data acquisition has been carried out most commonly at a single tumor level. The z-axis tumor coverage with such single level techniques depends on the number and configuration of CT detectors. For example, tumor coverage with a 4-detector row scanner (Lightspeed Plus; GE Healthcare Technologies, Waukesha, WI, USA) is 20 mm (four contiguous 5 mm axial images, or two contiguous 10 mm axial images), but this increases to 40 mm (eight contiguous 5 mm axial images, or four contiguous 10 mm axial images) for a 64-detector row scanner (VCT; GE Healthcare Technologies, Waukesha, WI, USA). Sequential data sampling is performed. At least one baseline nonenhanced image is required to allow detection of enhancement change following intravenous contrast medium administration. Data sampling rate and duration depend on a number of factors including the required perfusion parameter, the mathematical analysis method used, and other considerations such as tumor site and tumor size. Like DCE-MRI, a lack of consensus remains regarding acquisition technique, and mathematical analysis method to use in tumor perfusion assessment. This lack of standardization has been related in part to commercial implementation of tumor perfusion software packages based on different analysis methods, including unicompartmental analysis, Patlak analysis, and the modified distributed parameter model. The software themselves impose strict data acquisition requirements which has resulted in wide variation in acquisition techniques in clinical use.

First pass studies permit measurement of blood flow, blood volume, and mean transit time. A high temporal sampling rate of 1 acquisition per second for a duration of 45–65 s has been recommended for blood flow, blood volume, and transit time assessment. Delayed phase studies permit measurement of vessel permeability. This requires longer but less frequent data acquisition, in the order of 2–3 min with a sampling rate of up to 1 per 5 s. Hybrid acquisition techniques incorporating both first pass and delayed phase imaging are permitted by commercial software, and allow measurement of multiple perfusion parameters simultaneously. For example, blood flow, blood volume, mean transit time, and permeability can be assessed using the deconvolution/modified distributed

parameter model (Perfusion 3.0; GE Healthcare Technologies, Waukesha, WI, USA) or by compartmental/Patlak analysis (Body perfusion; Siemens Medical Solutions, Forchheim, Germany) by performing an acquisition with a high sampling rate (1/s) for the initial 60 s, and then a lower sampling rate (up to 1/5 s) for the next 60–120 s. The radiation dose of such studies will depend on the scan acquisition parameters and scan duration; however, an effective dose of 8 mSv has been quoted for a typical single level perfusion study (Lee *et al*., 2003).

In clinical practice the optimal data sampling rate and scan duration for colorectal tumor permeability measurement are still unknown. When colorectal tumor blood flow, blood volume, mean transit time, and permeability measurements calculated by the deconvolution/modified distributed parameter model were compared for three different acquisition durations of 45, 65 and 130-s, respectively, no differences were noted for mean tumor blood flow, blood volume, and transit time. However, significant differences in tumor permeability were noted between acquisitions of 45-s duration and 65 and 130-s duration, respectively, but not for acquisitions of 65 and 130-s duration (Goh *et al*., 2005a). This may appear counterintuitive as one may expect a longer acquisition to provide more reliable permeability measurement. However, tumor vessels are typically leaky to low molecular weight contrast agents. For example, up to 42% of contrast has been shown to leak out in the first pass in breast tumors, and significant amounts of contrast will have returned to the intravascular compartment by 2 min. The lack of significant difference between acquisitions of 65 and 130-s may be related in part to this phenomenon.

For anatomical sites, such as the liver, where excessive movement along the long axis of the body may occur because of respiration, breath-hold acquisitions are required to reduce misregistration and resulting mathematical modeling failures. Breath-hold up to 40-s is usually achievable, particularly with the assistance of oxygen breathing. For studies requiring a longer acquisition, for example, to assess permeability changes in colorectal metastases, multiple breathhold acquisitions can be performed, though care must be taken to ensure the same tumor level is examined during such acquisitions. More recently, data from rats have indicated that registration procedures to eliminate breathing motion can be successfully applied to liver perfusion studies, allowing longer acquisitions, and human data are awaited. Motion misregistration appears to be less of a problem for DCE-MRI hepatic studies because motion may be compensated for by the use of navigator technology developed for cardiac applications.

The most commonly implemented mathematical modelling methods for assessment of tumor vascularity at CT are unicompartmental analysis based on the Fick principle and modifications including Mullani–Gould formulation or slope method, Patlak analysis, and deconvolution/modified distributed parameter analysis. The Fick principle is one of the most straightforward methods of calculating tissue perfusion (flow per unit tissue volume). The Fick principle states that the amount of contrast taken up by an organ or tissue per unit time is equal to the arterial concentration minus the venous concentration multiplied by blood flow. Thus, perfusion can be calculated as follows: the tissue contrast concentration divided by the difference

in arterial and venous contrast concentration at time t. However, as this method requires determination of tissue, arterial and venous-time concentration curves in their strict form, this is generally impractical for CT.

Thus, methods such as Mullani–Gould formulation have been implemented. This is also known as the 'no venous outflow method'. By restricting measurements to the time prior to which contrast has begun to exit from the tissue or organ of interest, the venous term can be considered as zero, and the need for venous measurement is obviated. Perfusion is calculated by dividing peak tissue contrast concentration by peak arterial contrast concentration. A gamma variate fitting process has to be applied to the arterial-time curve to correct for recirculation, as failure to do so will lead to overestimation of the area under the curve, and a lower perfusion value. Other methods, such as the slope method have also been implemented in commercial software. This calculates perfusion from a shorter acquisition than with the Mullani–Gould method. Perfusion is calculated by dividing the maximum slope of the tissue enhancement curve by peak arterial enhancement. While the shorter acquisition ensures that the assumption of no venous outflow is met, this technique is innately sensitive to noise as this is a mathematical differentiation with respect to time of the Mullani–Gould formulation. These 'no venous outflow' methods also impose restrictions on the acquisition technique. Peak arterial enhancement should occur before the maximal rate of tissue enhancement. Therefore, a narrow contrast bolus is a necessary prerequisite for these techniques, and a small volume of intravenous contrast must be administered at a fast rate, typically 50 ml or less, at rates of 7 ml/s.

Patlak analysis has been used previously to model tracer kinetics in nuclear medicine studies, and has been adapted for analysis of permeability and blood volume (Patlak *et al*., 1983). This is a two-compartment model that describes the one-way transfer of freely diffusible contrast from the intravascular to the extravascular–extracellular compartment. These two compartments are assumed to be well-mixed. At any time point, the contrast concentration in tissue is deemed equivalent to the sum of the intravascular and extravascular concentration of contrast as denoted by the following equation: $c(t) = bv * b(t) + K * \int b(t).\, dt$, where 'c(t)' is the concentration of contrast within the tissue, 'bv' is the blood volume, 'b(t)' is the concentration of contrast in blood, and 'K' is the Patlak extraction fraction. Dividing the equation by 'b(t)' produces the linear equation: $c(t)/b(t) = rbv + K * \int b(t)\, .\, dt/b(t)$. By plotting this graphically to produce the Patlak plot, K, can be derived from the gradient of the slope of this line, and blood volume, bv, from the y-intercept. 'K' reflects both extraction and flow; thus, a disadvantage is that flow may affect permeability measurement. In some situations where vessel permeability is high, flow will be the limiting factor and K may approximate flow rather than permeability.

The adiabatic approximation of the distributed parameter model, also known as the modified distributed parameter model, describes the relationship between contrast in the intravascular and extravascular extracellular space, but takes into account the varying intravascular concentration gradient from the arterial inlet to venous

outlet. It is based on the model first proposed by Johnson and Wilson (1966). The capillaries within the tissue of interest are treated as a single compartment, around which the extravascular–extracellular space is regarded as a separate well-stirred compartment. The intravascular contrast concentration is dependent on both the axial position along the capillary and time, while the interstitial contrast concentration is dependent on time only. The original Johnson and Wilson model was of limited use, as its solution was expressed in the frequency domain. However, by using an adiabatic approximation to derive a closed form solution of the model in the time domain, a more workable model was produced for the cranial circulation. The resulting time domain solution for a mass of contrast per unit mass of tissue can be expressed as $Q_t = F \cdot C_a(t) * R(t)$, where R(t) represents the constrained impulse residual function. Both tissue contrast concentration (Q_t) and arterial contrast concentration $C_a(t)$ can be measured by CT. The constrained impulse residual function can be derived by deconvolution. From this blood flow, blood volume, mean transit time, and permeability can be derived, and this has been described in detail elsewhere (Lee *et al*., 2003).

It should be noted that measurements obtained using different modelling methods are not identical, nor necessarily interchangeable, and so caution must be applied in the comparison and interpretation of these measurements if the exact same methodology has not been used. For example, when colorectal cancer permeability and blood volume were compared for two different analysis methods, modified distributed parameter analysis and Patlak analysis, a coefficient of variation of 38% and 47% was noted for permeability and blood volume, respectively (Goh *et al*., 2007a). Measurements obtained using Patlak analysis were higher than for distributed parameter model, of the order of 1.34 times for permeability and 1.65 times for blood volume. The comparability of perfusion values obtained using the slope method and deconvolution has been investigated also at other sites. The slope method showed consistently lower perfusion values than the deconvolution method, but overall a good correlation was reported for perfusion measurements of lung nodules and spleen ($r = 0.86$ and $r = 0.90$, respectively) (Miles and Griffiths, 2003). However, the limitations of using correlation to assess agreement have been highlighted. Correlation examines the linear association between two variables and not the level of agreement between them. Correlation may be high in the face of considerable disagreement. For example, if one test consistently produced a result exactly twice that of the other, there would be perfect linear correlation despite disagreement of 100%. Thus, while these measurements show good correlation, the level of agreement has yet to be determined for these sites.

Dynamic Contrast Enhanced Magnetic Resonance Imaging

As with perfusion CT, a variety of DCE-MRI techniques are available for use in clinical practice, though the lack of commercially available quantitative software has limited widespread use of quantitativetechniques. The most commonly used sequences to assess tumor perfusion and permeability are $T2^*$ and T1 weighted sequences. Choice of sequence and sequence

parameters depend on anatomical coverage, and requirement for quantification. T2* weighted sequences (susceptibility weighted echoplanar spin echo or gradient echo sequences on conventional systems) are equivalent to the bolus tracking sequences used in perfusion CT. Typical parameters for a 1.5 T scanner are TR 30 ms, TE 20 ms, flip angle 40°, rectangular matrix 64 × 128, slice thickness 8 mm, one acquisition at a single level every 2 s for 2 min. Darkening of the signal intensity is detected, and the resultant signal intensity-time curve allows transit time, relative perfusion and blood volume to be defined. Measurements are relative rather than absolute for colorectal cancer, and other extracranial cancers, as direct measurement of the arterial input is not easily achievable. This is due to a combination of artefact from nonlaminar flow within large vessels, and the effects of high vascular permeability. Contrast media leakage into the extravascular space in the first pass produces T1 signal enhancing effects that counteract the T2* effects.

Gamma variate fitting of the signal intensity-time curve allows transit time to be obtained from the full width half maximum of the fitted curve, and relative blood volume to be obtained from the integral of the curve. Relative blood flow is determined from these measurements as blood flow = blood volume/transit time, as defined by the central volume theorem. Although a further parameter, the vessel tortuosity index, can be obtained from T2* data in the cranial circulation, this is not possible for extracranial tumors including colorectal cancer. This index is obtained from the difference between the integral of the total time series and integral of the gamma variate fitted first pass series, and reflects contrast retention in abnormal tumor vasculature. However, when there is significant leakage of contrast from the intravascular space (as occurs for low molecular weight agents in colorectal cancer), it is not measurable.

T1 weighted sequences (T1 weighted gradient echo, saturation recovery/inversion recovery snapshot sequences or echo planar sequences) provide information on microvessel perfusion, permeability, and extracellular leakage space. A 2D or 3D sequence can be used depending on the restrictions placed by software analysis. Typical parameters for a 2D sequence are TR 11 ms, TE 4.7 ms, flip angle 35°, matrix 192 × 256, one acquisition every 5 s for up to 8 min. Volume of coverage depends on tumor coverage needed. For colorectal cancer, this is typically 24 mm, consisting of 3 × 8 mm slices. Typical parameters for a 3D sequence are TR 7 ms, TE 1.5 ms, flip angle 30°, matrix 192 × 256, with a continuous acquisition for stationary structures, or one acquisition every 30 s if interval breathing is required between breathholds for up to 10 min. The volume of coverage depends on tumor coverage required. Typically 12–15 slices are obtained.

Contrast causes shortening of T1 relaxation and signal enhancement detection. The T1 enhancement curve has a typical appearance in tumors. There is a sharp upslope that predominantly reflects tumor perfusion, a maximal enhancement phase that reflects contrast concentration in the leakage space, and washout phase that reflects vessel permeability, and return of contrast from the extravascular compartment back to the intravascular compartment. Semi-quantitative measurements, that are straightforward to obtain, have been used to assess tumor vascular-

ity. These parameters have included curve shape, onset time, gradient of the upslope of the SI curve, maximum signal intensity, washout gradient, and area under the SI curve. However, the main limitation for widespread use is that they do not accurately reflect contrast concentration, and can be influenced by scanner settings and acquisition parameters making inter-patient comparison challenging.

Quantification is possible so long as appropriate phantom calibrations are performed, which enable the relationship between signal intensity and contrast agent concentration over the measured range of values to be determined. Signal intensity values from the dynamic acquisition are converted to contrast agent concentration values for each time point. Pharmacokinetic modeling can then be performed to obtain quantitative data. The most widely used is the general kinetic model modified from the Kety model (Kety, 1951; Tofts, 1997; Tofts *et al*., 1999). The contribution of intravascular contrast to tumor contrast is assumed to be negligible. The change in tumor contrast concentration over time is governed by the equation: $dC_{tumor}/dt = K_{trans}C_p - K_{ep}C_{tumor}$, where C_{tumor} and C_p represent the contrast concentration in the tumor extravascular extracellular compartment, and blood plasma compartment, respectively; K_{trans} represents the transfer constant between blood plasma and the extravascular extracellular compartment; and K_{ep} represents the rate of return of contrast between the extravascular extracellular compartment and blood plasma. K_{ep} is related to K_{trans}: $K_{ep} = K_{trans}/V_{e,}$ where V_e is the fraction of tumor volume occupied by the extravascular extracellular compartment. Thus, the concentration of contrast in tumor is determined by the blood plasma concentration curve, K_{trans} and $V_{e.}$ It has been noted that K_{trans} is a function of flow and permeability. More precisely, $K_{trans} = F * EF$ where F is flow and EF is the extraction fraction, or the initial fraction of contrast that diffuses into the extravascular extracellular compartment during the first pass as defined by $EF = 1 - e^{(PS/F)}$, where PS is the permeability surface area product and F is flow. When permeability is high, the extraction fraction, EF, approximates 1, and $K_{trans} = F$; thus, the behaviour of contrast is flow limited (as in the Kety model). Conversely, when permeability is low compared to F, the extraction fraction approximates PS/F and $K_{trans} = PS$, and the behavior of contrast is permeability limited. Further modeling approaches have attempted to separate the contributions of blood flow, volume, and permeability to the signal intensity change with DCE-MRI, but these approaches are currently too demanding for routine clinical use.

VALIDATION AND MEASUREMENT REPRODUCIBILITY

Previous studies have reported mean (standard deviation, SD) of primary colorectal cancer blood flow and blood volume measurements to be 91.1 (31.1) ml/100 g tissue/min, and 6.1 (1.3) ml/100 g tissue using distributed parameter analysis (Goh *et al*., 2006), in comparison to normal rectal measurements of 31 (15.5) ml/100 g tissue/min, 3.4 (1.6) ml/100 g tissue, respectively (Sahani *et al*., 2005). With DCE-MRI, mean (SD) perfusion index measurements of 11.4 (0.7) ml/100 g tissue/min have been reported (De Vries *et al*., 2001). These blood flow values

lie within the wide range from previous pooled data of 1.5–200 ml/100 g tissue/min (Vaupel, 2000).

Perfusion CT has been validated against a variety of techniques, including microspheres (Purdie *et al*., 2001; Cenic *et al*., 2000), xenon CT (Wintermark *et al*., 2001) and O_{15} labelled-H_20 PET (Gillard *et al*., 2000) in animals and humans. Whether or not these measurements truly represent tumor angiogenesis is less clear. Computed tomography and DCE-MRI measurements have been correlated against histological markers of angiogenesis in some tumors. For example, blood volume and permeability measurements assessed by perfusion CT have been correlated with microvessel density for colorectal cancer (Goh *et al*., 2008a), permeability has been correlated with microvessel density and vascular endothelial growth factor in renal cancer (Ueda *et al*., 2006), and semi-quantitative measurements including peak enhancement have been correlated with angiogenesis in lung cancer (Tateishi *et al*., 2002; Yi *et al*., 2004). Semiquantitative T1 kinetic parameters have been broadly correlated with microvessel density for colorectal (Tuncbilek *et al*., 2004), breast (Buckley *et al*., 1997), and cervical cancer (Hawighorst *et al*., 1998). However, other studies have found no such correlation (Li *et al*., 2005; Su *et al*., 2003). The exact reasons for this are unknown, but may reflect the spatial and temporal heterogeneity of tumor perfusion, method of histological analysis, observer variability of such analysis, and perfusion measurement selected.

Measurement reproducibility is generally acceptable for perfusion CT and DCE-MRI. A variation of 13.2–35% has been reported for perfusion CT in the cranial circulation of both animals and humans (Cenic *et al*., 2000; Nabavi *et al*., 1999). A similar level of measurement reproducibility has been noted in the extracranial circulation. For example, a variability of 14–24% has been reported in an animal tumor model using CT (Purdie *et al*., 2001), a coefficient of variation between 14% and 24% has been reported for colorectal cancer (Goh *et al*., 2006), and between 9% and 26% for lung cancer (Ng *et al*., 2006) in humans at CT. In general, the measurement variability of DCE-MRI techniques in the extracranial circulation is greater, partly reflecting the inability of these techniques to adequately compensate for input function, as the vascular concentration of MRI contrast medium is not easily measured. The reproducibility of K_{trans}, reflecting blood flow, for a variety of extracranial cancers, has been reported as 26% (Galbraith *et al*., 2002). Previous studies of observer variability have demonstrated that inter-observer variability is greater than intra-observer variability, though overall this is acceptable for therapeutic assessment. Intraclass correlation coefficients of 0.73–0.89 reflecting inter-observer agreement have been noted for cerebral perfusion measurements obtained using CT (Fiorella *et al*., 2004). Similarly, intraclass correlation coefficients of 0.80–0.99 have been noted for colorectal cancer measurements (Goh *et al*., 2005b), while intraclass correlation coefficients of 0.97–0.99 have been noted for lung cancer using CT (Ng *et al*., 2006).

Whether or not measurement variability will impact on therapeutic assessment remains to be seen. However, of the anti-angiogenic and anti-vascular drugs that have undergone or are undergoing clinical evaluation currently, including bevacizumab (Avastin; Genentech, CA, USA), PTK787/ZK 222584 (Vatalanib; Novartis, NJ, USA

and Schering AG, Berlin, Germany), and combretastatin (OXiGENE, NY, USA), measurement variability appears to be within the levels of expected therapeutic change. For example, in a phase I study of single agent bevacizumab in rectal cancer, a mean change in perfusion CT acquired blood flow in the order of 40% was noted (Willett *et al*., 2004); in a phase I study of PTK 787/ZK 222584 in advanced colorectal cancer, a mean change in DCE-MRI acquired $K_{I,}$ the bi-directional transfer constant of 58% was noted across all doses 48 h post-drug administration (Morgan *et al*., 2003). A phase I study of combretastatin in a variety of tumors showed a mean change in DCE-MRI acquired K_{trans} in the order of 37% (Galbraith *et al*., 2003).

CLINICAL UTILITY

Distinction Between Benign and Malignant Disease

Although perfusion techniques have been advocated predominantly for therapeutic assessment, these techniques may have a role as a diagnostic tool. Perfusion measurements have been noted to be significantly different for malignant and benign tissue. For example, blood flow, blood volume, and permeability measurement using perfusion CT have been shown to be significantly higher for rectal cancer than normal rectum (Sahani *et al*., 2005). Likewise, DCE-MRI perfusion index measurements (PI; maximum of the arterial T1 signal intensity-time curve divided by the maximum slope of the tumor T1 signal intensity-time curve), which reflect tissue perfusion and permeability, have been shown to be significantly higher for rectal cancer than normal tissue (skeletal muscle) (Rudisch *et al*., 2005). Similarly, blood flow, blood volume, and permeability measurements have been shown to be substantially higher for rectal cancer than normal skeletal muscle using perfusion CT (Goh *et al*., 2006).

Quantitative perfusion Computed tomography measurements may be able to differentiate diverticulitis from primary colorectal cancer. Computed tomography is currently the imaging modality of choice for assessing diverticulitis; however, diagnostic confusion between diverticulitis and cancer is not uncommon. Both colorectal cancer and diverticulitis produce abdominal symptoms such as change in bowel habit and abdominal pain, and imaging features show considerable overlap at CT. Indeed, a previous study showed that when discriminatory CT morphological criteria alone were applied prospectively in these patients, 51% of cases required further evaluation to exclude a cancer (Chintapalli *et al*., 1999). Blood volume, blood flow, and permeability measurements at perfusion CT have been found to be significantly different between patients with cancer, diverticulitis and inactive diverticular disease, with cancer having the highest blood volume, flow, and permeability (Figure 12.1), and inactive diverticular disease the lowest blood volume, flow, and permeability (Goh *et al*., 2007).

This is similar to data obtained for lung nodules (Zhang and Kono, 1997), and likely reflects the proangiogenic nature of cancer, and the vasodilatation, increased local blood flow, and vascular permeability related to cytokine release in inflammation. Blood volume and flow had sensitivity of 80% and specificity of 70% and 75% for differentiating cancer from diverticulitis, which was better than that achieved using

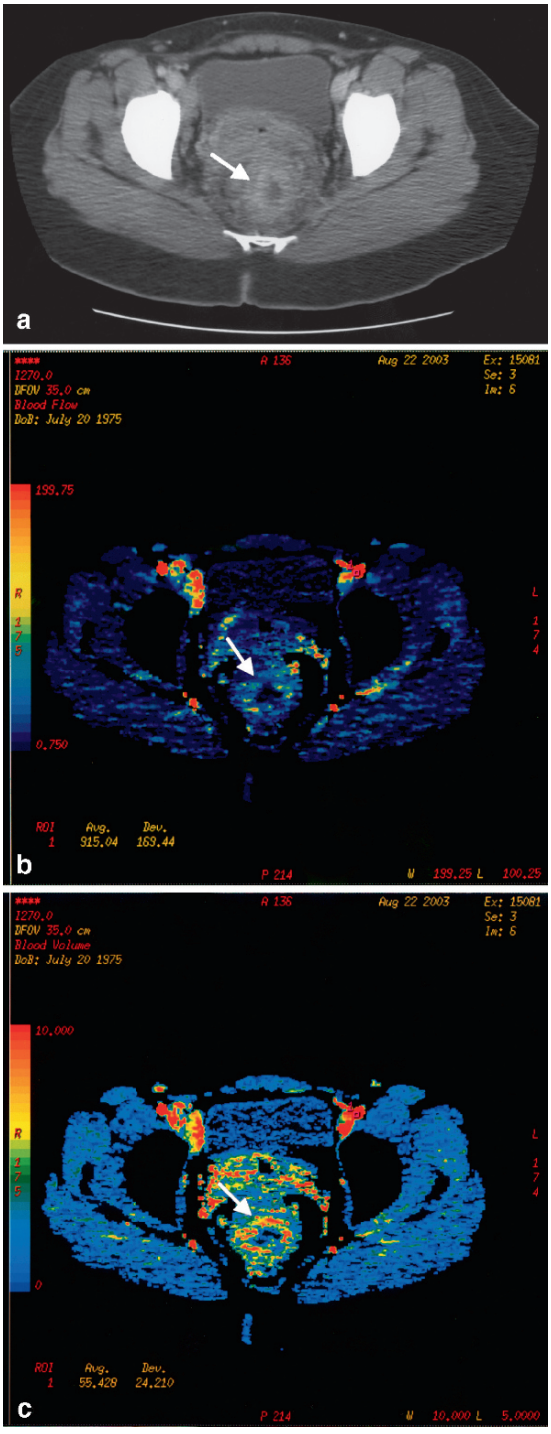

FIGURE 12.1. Contrast enhanced axial CT image (a) demonstrating a rectal cancer (arrow) with corresponding blood flow (b), and blood volume (c) perfusion CT parametric maps showing the heterogeneous distribution of vascularity

most standard morphological criteria in this study, apart from the presence of pericolonic nodes. Thus, there may be a role for such perfusion measurements in this clinical scenario, particularly for difficult cases where morphological features are unhelpful. To date there have been no quantitative DCE-MRI studies addressing this issue, nor have there been any published studies on assessment of local disease relapse using quantitative DCE-MRI or perfusion CT. There have been some semi-quantitative DCE-MRI studies that have suggested that enhancement change may be capable of distinguishing disease relapse from post-treatment fibrosis (Muller-Schimpfle *et al.*, 1993; Kinkel *et al.*, 1996), though other studies have shown no such ability, as inflammation as a consequence of radiotherapy may cause similar enhancement changes (Blomqvist *et al.*, 1998).

Assessment of Chemoradiotherapy

Radical radiotherapy is being given increasing importance in combination with chemotherapy. Novel drugs such as monoclonal antibodies and vascular targeting drugs are also being assessed in combination with standard treatment. Thus, a better understanding of tumor perfusion changes with radiation or chemoradiation is becoming essential, for example, to guide drug scheduling. Previous *in vitro* studies of the acute effects of ionizing radiation on tumor vasculature have shown that the tumor response to radiation is regulated by tumor endothelial cell apoptosis (Garcia-Barros *et al.*, 2003; Pena *et al.*, 2000). Up-regulation of vascular endothelial growth factor (VEGF), either directly or through activation of endothelium hypoxia inducible factor (HIF-1), has also been

reported in various cancer cell lines after ionizing radiation, which may cause further neovessel formation (Moeller *et al*., 2004). Acutely, an increase in vascular permeability and blood volume is noted as a consequence of endothelial cell damage and inflammation, and possibly due to further neovessel formation. This has been shown *in vivo* in a heterogeneous group of solid human tumors using perfusion CT (Harvey *et al*., 1999). More chronically a decrease in vascular permeability is seen due to basement membrane thickening, extracapillary fibrosis, and endothelial damage. A reduction in microvessel functionality from thrombosis and obliteration of the vessel lumen also occurs. Few studies have evaluated the vascular effects of standard chemotherapeutic agents alone. It is recognized that standard chemotherapeutic agents used in clinical practice may have an anti-angiogenic effect; however, a recent study has shown that platinum based agents have no significant acute vascular effect using quantitative DCE-MRI (Lankester *et al*., 2005).

Studies of primary colorectal cancer have been performed following chemoradiation, rather than following radiation alone; thus, observed vascular changes are due to the combined anti-vascular effects of radiation and the chemotherapeutic agent used. The majority of perfusion CT and DCE-MRI studies have focused on the overall vascular change following completion of treatment. Patient numbers in these studies have been typically small. For example, perfusion CT has been performed in nine patients before and 1–2 weeks following completion of chemoradiation. Mean blood flow was found to be significantly lower following treatment, although changes were not homogeneous: in two patients an increase in blood flow was noted (Sahani *et al*., 2005). Similarly, DCE-MRI was performed in 16 patients undergoing preoperative chemoradiation, and mean k_{trans} was noted to be lower following treatment (George *et al*., 2001). One study assessed the weekly changes in vascularity during chemoradiation in 11 patients using DCE-MRI. A perfusion index (PI) was measured at baseline, and repeated once weekly during treatment for 4 weeks. Significant increases in mean PI were seen in the first 2 weeks of treatment indicating an increase in vascular perfusion and permeability, with a reduction in mean PI by week four of treatment though mean values remained above baseline values (De Vries *et al*., 2000).

These measured changes provide some insight into tumor physiology, but what about clinical utility? Studies have attempted to use these measurements to identify patients who may or may not respond to treatment. Results have suggested that it may be possible to distinguish responders from nonresponders from baseline perfusion values. Colorectal tumors with a higher baseline blood flow show a poorer response to chemoradiation, and this has been demonstrated at perfusion CT (Sahani *et al*., 2005), and DCE-MRI (De Vries *et al*., 2001), but not all studies have been in concordance. For example a study has shown the opposite effect: higher initial k_{trans} values predicted for tumor response to chemoradiation (George *et al*., 2001). However, in this study the initial DCE-MRI measurements were performed 10–12 weeks following commencement of chemotherapy (5 FU and mitomycin C), and these initial k_{trans} measurements are unlikely to reflect true baseline nontreated values. These studies were performed with

small patient numbers, and it remains to be seen if these findings are true in larger series.

Assessment of Anti-angiogenic Drugs

Therapeutic assessment has been the main reason for the clinical proliferation of perfusion techniques. Drugs that disrupt the angiogenic pathway provide a more targeted tumor specific approach than current chemotherapeutic drugs, are potentially less toxic, and thus are an attractive therapeutic option. Angiogenesis is a relatively tumor specific process, though it is also evident in menstruation and wound healing, and has been shown to be essential for tumor growth. Tumor angiogenesis occurs in response to an increased need for oxygenation and nutrient supply. Hypoxia inducible factor (HIF-1) induced expression of VEGF and VEGF receptor binding triggers the angiogenic cascade causing an increase in vascular permeability that can be detected with imaging. This increase in permeability is necessary to allow extravasation of plasma proteins, and alteration of the extracellular matrix to generate a favorable environment for new vessel formation.

Anti-angiogenic drugs that have been assessed in colorectal cancer include bevacizumab (Avastin, Genentech, San Francisco, CA, USA), a monoclonal antibody targeted at VEGF, an important component of the angiogenic cascade, and PTK787/ZK 222584 (Vatalanib; Novartis Pharmaceuticals Corporation, NJ, USA and Schering AG, Berlin, Germany), a multiple VEGF receptor inhibitor that blocks the activity of all known VEGF receptor tyrosine kinases. Bevacizumab in combination with conventional chemotherapy has been shown to improve survival in a Phase III study of metastatic colorectal cancer and has been licensed for use in colorectal cancer (Hurwitz *et al*., 2004). Bevacizumab has been generally well tolerated, although hypertension, epistaxis, proteinuria, and thrombosis have provided safety concerns. PTK787/ZK 222584 has yet to be licensed for use.

Although size change has been shown to be unrepresentative for response assessment of these drugs, traditional response methods remain the ‘gold standard’ for drug licensing purposes, and perfusion measurements are unlikely to replace these in the near future. However, perfusion techniques have a role in early phase clinical studies to demonstrate an anti-vascular effect and to define a biologically active dose. Drug pharmacokinetics and drug toxicities are typically assessed in Phase I studies. In particular, drug toxicities are used to define a dose to take forward for further evaluation. With their relatively low toxicity and wider therapeutic window this approach may be less valid for antiangiogenic and anti-vascular drugs. By demonstrating an anti-vascular effect during dose escalation, perfusion imaging has been able to define a biologically active dose that is lower than the dose limiting toxicity and maximum tolerated dose (Galbraith *et al*., 2003).

Perfusion imaging may provide early proof of principle of drug action. For example, perfusion CT was able to demonstrate that bevacizumab had direct antivascular effects in rectal cancer. A significant reduction in blood flow was demonstrated 12 days following single agent administration, which correlated with a decrease in microvessel density (Willett *et al*., 2004). Perfusion imaging may also provide an early indication of drug effect, and support

go-no-go decisions on new compounds. Whether such vascular changes reflect eventual outcome remains to be seen; however, data from a phase I trial of PTK787/ZK 222584 for metastatic colorectal cancer have been promising. DCE-MRI was able to demonstrate that a decrease in transfer constant of greater than 40% at day 2 post-drug administration could predict for non-progression of disease (Thomas *et al*., 2005).

Assessment of Hepatic Metastatic Disease

Hepatic assessment has provided certain challenges for perfusion imaging as the dual supply of the liver has to be taken into account in quantitative evaluation. Up to 75% of normal hepatic blood supply is derived from the portal venous system, and 25% from the hepatic artery. However, hepatic metastases derive most of their blood supply from the arterial system, and this is exploited in perfusion imaging. Perfusion imaging has been used for assessment of therapeutic effect, as a prognostic tool, and for the detection of occult disease. It has been established that a global increase in hepatic arterial perfusion occurs with overt metastatic disease. This has been demonstrated with slope-ratio analytic methods using perfusion CT (Miles *et al*., 1993; Blomley *et al*., 1995), and 3D T1 weighted DCE-MRI (Totman *et al*., 2005). The hepatic perfusion index (HPI) or ratio of arterial to whole liver perfusion has been used as a measure of this increased vascularity.

In addition to global changes in hepatic perfusion, individual metastases also show evidence of increased perfusion particularly at the lesion rim, and again this has been demonstrated using perfusion CT and DCE-MRI. This increased rim enhancement has been shown to correlate with the degree of neovascularization and degree of peripheral desmoplasia previously (Semelka *et al*., 2000). This is exploited in therapeutic response assessment. The degree of rim perfusion at CT may also provide some prognostic information, and has been correlated with survival over a year (Miles *et al*., 1998).

Studies of perfusion imaging for the assessment of occult metastatic disease have shown interesting results. The presence of micrometastases alters hepatic perfusion patterns. There is an increase in hepatic resistance, which may be related to the presence of microthrombi within portal venules, or possibly due to release of a tumor factor. In rats inoculated with micrometastases, a reduction in portal perfusion of 34% was identified at perfusion CT even though these were not visible on conventional imaging (Cuenod *et al*., 2001). Similar changes have been demonstrated in humans with occult metastases at CT (Leggett *et al*., 1997), and Doppler ultrasound (Leen *et al*., 1993). However, these findings have yet to be corroborated in any larger study.

Primary Tumor Perfusion and Development of Metastatic Disease

Preliminary data on primary colorectal perfusion as a prognostic indicator have been promising. Although 70% of cases of primary colorectal cancer are treated with curative intent, up to 30% of these patients will develop metastatic disease within 3 years of diagnosis. Histological studies of primary colorectal microvessel density (MVD) have demonstrated that there is a significant difference in MVD in patients with or without metastatic disease, with a higher MVD in

metastatic patients, suggesting that angiogenesis is important for development of metastatic disease (Tomisaki *et al*., 1996). Perfusion CT performed at presentation may be able to identify patients who subsequently develop metastatic disease. Significant differences in mean perfusion were found between patients who developed metastatic disease and patients who remained disease free at 3 years follow up. Patients who developed metastatic disease had a lower blood flow and permeability at presentation (Goh *et al*., 2008b). However, the number of patients in this study was small. Once patients had been excluded, for example, because of lack of imaging follow up, only 32 patients remained from the initial cohort. It remains to be seen if results are true in a large-scale study. There has been no assessment of the prognostic value of quantitative colorectal cancer measurements using DCE-MRI in the same clinical situation to date.

WHICH IMAGING TECHNIQUE SHOULD BE USED?

The question that is often asked is, 'Is one technique better than the other?' To date no studies have compared the performance of perfusion CT and DCE-MRI for tumor assessment, in particular, for colorectal cancer. A study has compared the performance of perfusion CT and DCE-MRI for the evaluation of solitary pulmonary nodules; and concluded that there was no significant difference in performance of the two techniques (Kim *et al*., 2004). In reality, choice of perfusion CT or DCE-MRI for assessment of colorectal cancer is based on several factors: local expertise and availability, need for quantification, perceived radiation burden, and site of disease. Although quantitative DCE-MRI has become increasingly established as a surrogate for angiogenesis, quantification is not straightforward. Artefacts also remain an issue. For example, although less relevant for primary colorectal cancer, phase encoded artefacts arising from vascular pulsatility, and exaggerated by concentrated contrast medium, can render DCE-MRI uninterpretable. Computed tomography remains the most commonly used modality for cancer imaging, and the availability of truly quantitative commercial software that is straightforward to use may be a major determinant of its future use, as more data become available. Multicenter assessment is also more easily achievable in comparison to DCE-MRI, where the quality assurance challenges are greater.

CHALLENGES FOR PERFUSION IMAGING

Several challenges lie ahead for colorectal cancer perfusion imaging. Currently used techniques have been criticized for their limited tumor coverage. Tumor vascularity is typically heterogeneous, and whether assessment of such a small tumor volume truly represents the vascularity of tumor as a whole has been questioned. To counter this, volumetric imaging techniques for example, are now being applied for perfusion CT to lung cancer (Ng *et al*., 2006). These volumetric techniques enable the whole tumor to be encompassed. While quantitative measurements of permeability and blood volume could only be obtained,

previously CT technology has reached the point where volumetric scanning can be performed fast enough to achieve the temporal resolution required for blood flow evaluation. Clinical utility is being evaluated.

Secondly, concerns have been raised regarding the radiation burden imposed by perfusion CT. Attempts at dose reduction have been made. Scanning at 80 kV rather than 120 kV has been implemented at some sites, e.g., brain, lung, extracranial head, and neck. This has the dual advantage of reducing dose, and optimizing absorption of X-rays for iodine. For example, the volumetric technique described above for lung cancer is performed at 80 kV and confers an effective dose of 7.5 mSv (Ng *et al*., 2006). Low milliamperes, (as low as 60 mAs), also have been used without compromising the quality of perfusion data. Abdominal studies confer a higher radiation burden, but again attempts are being made to reduce dose at this site. A study has shown the feasibility of diagnostic abdominal scanning at 90 kV without significant reduction in diagnostic quality (Funama *et al*., 2005), and this is promising for the application of lower kilovolt perfusion techniques for colorectal cancer perfusion assessment.

Thirdly, validation is incomplete. Further validation is needed particularly of permeability and blood volume measurements obtained using low molecular weight tracers, though this may not be as straightforward as for blood flow measurement. For example, permeability is dependent on molecular size and, therefore, relatively specific for the tracer used. Fourthly, further steps need to be taken to incorporate perfusion imaging with other techniques to provide a more global tumor assessment. While this has occured in clinical practice with MRI, perfusion CT has lagged behind. For example, perfusion CT can be easily incorporated with 18-flurodeoxyglucose - PET-CT to provide assessment of tissue perfusion and glucose utilization, or indeed with any other tracer (Miles and Griffiths, 2003); however, this has yet to gain widespread acceptance. Assessment of tumor vascularity at CT colonography is a promising area for development. Qualitative assessment of tumor vascularity has been shown to be possible using 'vascular views' following contrast enhancement, which may be helpful in delineating cancer particularly in the unprepared colon (Iinuma *et al*., 2005). There is potential for quantitative assessment to be incorporated with this technique, aiding diagnosis and prognostication.

Finally, standardization is required. Attempts have been made for DCE-MRI assessment. The variability of semi-quantitative analysis and its dependence on multiple factors has been recognised, which has led to a consensus that quantitative methodology should be used for tumor assessment. In particular, k_{trans} assessment has been advocated, though some controversy remains over the correct modeling method. Perfusion CT is still playing 'catch up', though some leading exponents have suggested possible methods including the use of a standardized perfusion value. The best way forward remains to be seen.

In conclusion, quantitative assessment of colorectal cancer perfusion provides useful information. As a surrogate maker of angiogenesis, perfusion imaging has clinical util-

ity not simply for therapeutic assessment, but also shows promise as a diagnostic and prognostic tool for colorectal cancer.

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C. Prognosis

13
Positron Emission Tomography and Colorectal Cancer

Ur Metser

Colorectal cancer (CRC) is the third most common cancer in the United States with > 135,000 cases reported every year and a life-time risk of 5–6%. It is the second leading cause for cancer-related death in the western world. Although 80% of CRC cases are sporadic, in 20% of patients a hereditary predisposition exists. Several genetic mutations have been implicated in an increased risk for developing CRC. In familial polyposis coli, there is a mutation of the adenomatous polyposis coli (APC) gene on chromosome 5. Mutations in the genes responsible for repair of mismatched DNA base pairs (mismatch repair genes) are the major cause of cancers in hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome), the most common hereditary form of CRC, accounting for up to 5% of CRC cases (Giardiello *et al*., 2001). Patients with inflammatory bowel disease are also at an increased risk for the development of CRC, up to 2–8 times greater than the risk for the general population. This risk is related to the duration and anatomic extent of inflammatory disease, and coexistence of primary sclerosing cholangitis (Vagefi and Longo, 2005). Population studies have associated advanced age, certain diets (low fiber, high fat, and red meat intake), smoking, alcohol consumption, and obesity with the development of CRC; however, a cause and effect link has not been proven for these factors.

There is compelling epidemiological, clinicopathological and genetic evidence for an adenoma-carcinoma sequence in the development of most CRC's. The adenoma-carcinoma sequence refers to the development of CRC from adenomatous polyps. The likelihood of malignancy developing in an adenoma is directly related to its size, volume of villous tissue, and the severity of epithelial dysplasia. Multiple underlying molecular and genetic changes along the adenoma-carcinoma sequence have been identified. For example, an imbalance in genomic DNA methylation may lead to oncogene activation (hypomethylation) and silencing of tumor suppression genes (hypermethylation) (Hardy *et al*., 2000). The average estimated "dwell time" for an adenoma to transform into cancer is 10–15 years. However, not all adenomas progress to carcinomas, some may even spontaneously regress. Furthermore, many researchers believe that *de novo* carcinogenesis is a plausible alternate pathway to CRC development (Watanabe and Muto, 2000).

PROGNOSIS AND PRINCIPLES IN THERAPY

The prognosis of patients with CRC is related to the degree of tumor penetration through the bowel wall, and presence or absence of lymphatic spread and systemic metastatic disease. These prognostic factors are incorporated in the commonly used staging classifications, the TNM classification and Dukes classification (Tables 13.1 and 13.2). Prognosis is related to stage and is depicted in Table 13.2 (Winawer *et al*., 1997).

Stage I disease (Dukes A) is treated surgically with excellent outcome. In Stage II disease (Dukes B) surgical resection is highly effective for localized disease; however, more than one quarter of Dukes B patients develop recurrence and die from the disease. Adjuvant chemotherapy in patients with Dukes B disease is controversial with conflicting results in different studies (IMPACT B2, 1999). Certain patients with stage II CRC may be at a higher risk for recurrence; specifically, patients with tumor adherent to adjacent organs and patients presenting with complete obstruction or perforation. Recently, gene expression profiling has been shown to be able to predict recurrence in Dukes B patients, thus further stratifying these

TABLE 13.1. TNM staging system for CRC.

T: tumor	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*
T1	Tumor invading submucosa
T2	Tumor invading muscularis propria
T3	Tumor invading subserosa or pericolic/ perirectal fat
T4	Tumor perforating visceral peritoneal or adjacent organs
N: regional nodes	
Nx	Regional nodes cannot be assessed
N0	No regional node metastases
N1	One to three positive nodes
N2	Four or more metastatic nodes
M: distant metastases	
Mx	Distant metastases cannot be assessed
M0	No distant metastases
M1	Distant metastases present

TABLE 13.2. Modified Dukes staging system for CRC.

Stage	Details	5-YSR
A	Limited to submucosa & muscularis mucosa	90%
B1	Extends to muscularis mucosa	60–75%
B2	Extends through muscularis mucosa; no nodal spread	
C1	Within bowel wall & involves lymph nodes.	29–69%
C2	Extends through bowel wall & involves lymph nodes	
D	Distant metastases	5%

5YSR = 5-year survival rate.

patients into higher and lower risk groups. This may prove to have therapeutic implications (Wang *et al*., 2004).

Positive lymph nodes, i.e., Dukes stage C, increase the likelihood for tumor recurrence to 60% within 5 years. The number of lymph nodes involved affects prognosis, with a better survival for patients with less than four involved nodes. Postoperative chemotherapy reduces the recurrence rate to 40–50% (Wolmark *et al*., 1999). Adjuvant radiation therapy may have a role for patients with local residual disease after surgery. In advanced rectal cancer (> T3), adjunctive postoperative chemoradiation improves survival. However, due to bowel toxicity associated with postoperative radiation, preoperative radiation therapy has been attempted with improved local control, relatively low toxicity, and a 28% pathologic complete response rate, defined as absence of identifiable cancer cells in the surgical specimen (Mohiuddin *et al*., 2006).

Patients with hepatic and pulmonary metastases from CRC may benefit from aggressive surgical therapy. Resection of hepatic colorectal metastases has been shown to be a safe procedure with < 3% mortality. It may produce long-term survival (5-year survival rate > 33%) and even cure. A single institution's report on > 1,000 consecutive hepatic resections due to metastatic CRC has documented a 10-year survival rate of 22%. Of several factors tested to determine long-term outcome of resection of hepatic metastases in CRC, positive surgical margins and presence of extrahepatic metastatic disease are the strongest indicators for failure of a surgical approach and are considered contraindications to liver resection. In recent years, for patients with nonresectable hepatic metastases, radiofrequency ablation has emerged as a safe technique (major morbidity ~ 2%; mortality < 1%) that may provide long-term tumor control (Solbiati *et al*., 2001).

The role of neoadjuvant chemotherapy for patients with multiple (five or more) bilobar hepatic metastases is still being studied. In one recent study, such an approach may show a survival benefit and reduce the number of extended hepatectomies performed (Tanaka *et al*., 2003). Resection of both hepatic and pulmonary metastases secondary to colorectal cancer in highly selected patients may result in long-term survival, with 55% of patients remaining disease-free 5 years after surgery in one series. Thoracic lymph node involvement and elevated carcinoembryonic antigen levels (> 5 ng/ml) before pulmonary metastasectomy are associated with reduced survival.

IDENTIFICATION OF PRIMARY COLONIC LESIONS WITH 18F-FLUORODEOXY-GLUCOSE- POSITRON EMISSION TOMOGRAPHY TOMOGRAPHY

The incidence of colonic adenomas rises with age. The malignant potential of adenomas of the colon and rectum varies with size, histological type, and grade of epithelial atypia. Adenomas < 1-cm in diameter bear < 1% probability for cancerous transformation. Tumors with villous morphology and severe epithelial atypia are at a much higher risk for malignant transformation. Early identification and removal of colonic polyps have been shown to reduce the incidence of colonic

cancer (Winawer *et al*., 1997). A variety of screening modalities exist, including occult fecal blood, endoscopy, barium enemas, and recently virtual colonoscopy (CT colonography). Computed tomography colonography is usually performed with low-dose multidetector CT, although various other protocols exist. Computed tomography data sets are reconstructed into two and three-dimensional images of the colon. A meta-analysis of 2,610 patients has shown that CT colonography has a sensitivity of 86% and specificity of 86% for detection of polyps of medium and large size (Halligan *et al*., 2005). False-negative results may be due to small polyps, flat lesions, or due to inadequate bowel preparation (Park *et al*., 2005). Although controversial for polyp screening, the use of CT or magnetic resonance imaging (MRI) based colonography in patients with incomplete colonoscopy is becoming an accepted examination.

Currently, FDG-PET is not considered a screening modality for colonic polyps. However, multiple reports have shown that incidental colonic polyps may be identified with FDG-PET (Drenth *et al*., 2001) (Figure 13.1). In a prospective study on 100 patients with suspected focal lesions on sigmoidoscopy or barium enema, FDG-PET detected > 50% of all polyps identified on colonoscopy. The sensitivity of FDG-PET increased with adenoma size (21%, adenomas 1–5 mm; 47%, 6–10 mm; and 72%, > 11 mm). The sensitivity of FDG-PET also increased with the grade of dysplasia (33%, low grade; 76%, high grade; and 89%, carcinomas). The overall specificity was 84% (van Kouwen *et al*., 2005).

Aside from size and grade of dysplasia in individual lesions, FDG-PET may be limited in identifying colon cancers of mucinous subtype. This may be attributed to the hypocellularity of these tumors, resulting in overall low FDG uptake. The sensitivity of FDG-PET in identifying primary colon cancers as a whole is 87–100%, as compared with 56–59% for mucinous tumors (Berger *et al*., 2000). Another common obstacle in identifying focal colonic uptake is high-level physiological uptake of FDG in the large bowel. This uptake varies between individuals and inter-individually on consecutive studies. The precise etiology for increased physiological uptake of FDG in bowel is unknown, although several factors have been implicated including uptake by lymphoid tissue in the cecum, uptake by smooth muscle in bowel wall, swallowed secretions, or excretion and intraluminal concentration of FDG. A single study on

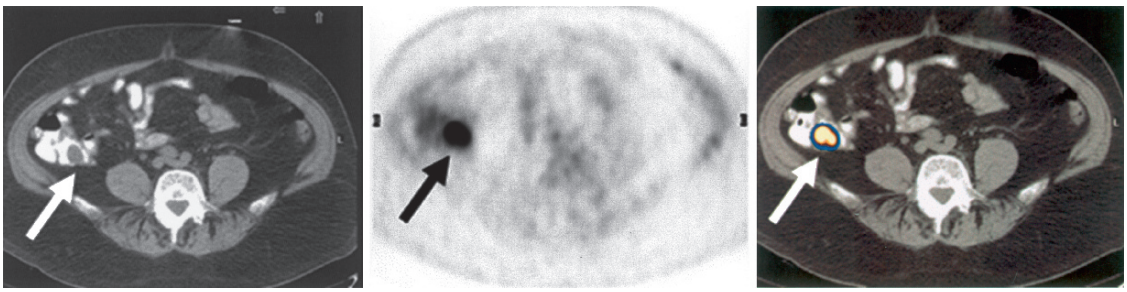

FIGURE 13.1. PET/CT performed on patient with lymphoma after therapy. Axial image (CT on left, PET in middle and fused PET/CT on right) shows incidental focal FDG uptake is seen in right colon, corresponding to a soft-tissue attenuating polyp on CT. Histology revealed a villous adenoma

physiological FDG uptake in the gastrointestinal tract was performed on rats after administration of omeprazole, which was effective in decreasing FDG uptake in the small intestine and colon (Yamamoto *et al*., 2004). However, no large-scale clinical trials to date have examined the effect of omeprazole or other drugs on physiologic uptake of FDG in the human colon.

New methodologies for polyp detection screening are continuously evolving with higher resolution imaging, and acquisition protocols are being introduced. Recently, a whole body PET/CT colonography protocol has been suggested by Veit *et al*. (2006a). This protocol has not yet been tested on a large volume of subjects; however, its main limitation as a screening modality may be patient radiation exposure. It is estimated that the effective dose per whole body PET/CT examination is ~ 25 mSv. This radiation dose may be unacceptable for screening purposes.

STAGING: LOCAL TUMOR, LYMPH NODE, AND DISTANT METASTASES

The two accepted staging methods for CRC are based on local tumor staging, referring to depth of invasion into bowel wall and adjacent organ involvement, lymph node staging, and presence or absence of distant metastases. Because there are no lymphatics in the lamina propria, carcinoma confined to the mucosa will not metastasize. Invasive CRC is therefore defined as tumor penetrating at least into the submucosa. Once in the submucosa, lymphatic spread of tumor is possible. Metastatic disease to regional lymph nodes usually follows a predictable path with extension to pericolic or perirectal lymph nodes and subsequently to central nodes along vascular trunks. There is a direct relationship between N-stage and patient survival (Shida *et al*., 1992). Distant metastatic disease may occur to the liver, lung, peritoneum, adrenal glands, ovary, bone, or brain. The most common metastatic site from CRC is the liver, presumably due to portal venous drainage of the colon. However, rectal carcinomas may show extra-hepatic hematogeneous metastases without liver metastases, due to the dual venous drainage system of the rectum (to the portal venous system and systemic venous system).

Hepatic metastases are present in 15–25% of patients at diagnosis (Fong *et al*., 1996) and in 25–50% within 3 years of diagnosis, following resection of the primary tumor. In approximately half of these patients, metastatic disease is confined to the liver, and 20% of all patients who die of metastatic colorectal cancer have metastases limited to the liver. Hepatic resection for CRC liver metastases remains the only potential curative option for these patients, with cure obtained in ~ 25% of cases (Adson, 1987). Traditionally, hepatic resection for CRC liver metastases was supported only if there was a maximum of three liver lesions, clear margins of 10 mm, and absence of extrahepatic disease. Today, novel approaches such as preoperative portal vein embolization and staged resection along with neoadjuvant chemotherapy have enabled resection of bilobar disease. Ablative therapy, such as radiofrequency ablation may be used in adjunct with hepatic resection to achieve a macroscopically tumor-free liver. *En bloc* resection of the inferior vena cava or hepatic veins with reconstruction and concomitant resection

of hepatic pedicle lymph node metastases are technically feasible. Resection of limited pulmonary metastases in addition to hepatic resection is also possible (Khatri *et al*., 2005). However, detailed knowledge of extent and location of liver metastases and presence of extra-hepatic disease is crucial for accurate patient selection and therapy planning. Despite optimal surgery, two-thirds of patients may develop hepatic recurrence after hepatic resection for metastatic disease. However, even in these patients, there appears to be a 12-month survival advantage when compared to patients in whom no treatment was offered despite resectable metastases confined to the liver (Scheele *et al*., 1990). In some of these patients, repeat resection of liver metastases is often feasible.

Peritoneal spread is encountered in ~ 7% of patients at primary surgery, in 4–19% of patients during follow-up and in 40–80% of patients who die from CRC. In up to a quarter of patients with metastatic disease, the peritoneal cavity may be the only site of metastatic disease. Peritoneal spread is thought to arise due to direct invasion of the bowel wall by an invasive cancer, or iatrogenically during surgery, due to embolization or escape of tumor cells from lymphatics, the bowel lumen, or due to tumor spillage during surgery to the peritoneal cavity. Cytoreductive surgery and adjuvant intraperitoneal chemotherapy have been shown to be efficacious in selected patients with resectable peritoneal carcinomatosis due to CRC (Kopper *et al*., 2006).

Conventionally, preoperative staging of patients with CRC is performed with CT, with an advantage to MRI in local staging of rectal tumors. Recently, multi-detector CT performed with portal venous or arterial and portal venous phase enhancement has been shown to be a relatively accurate modality for local staging (T-stage), with an overall accuracy of 57–83%. Thin collimation multiplanar reconstructions have an important contribution in correct local staging of primary tumor and regional lymph nodes. Although traditionally the anatomical imaging criteria for lymph node metastases are based on size (≥1-cm in short-axis diameter), some authors advocate a cluster of three or more regional nodes, regardless of their size, as suggestive of lymph node metastases (Furukawa *et al*., 2006).

Few studies exist on the performance of FDG-PET in preoperative staging of CRC. FDG-PET lacks the anatomic resolution for T-staging. A single innovative study has shown that PET/CT colonography is technically feasible. Correct T-staging was achieved for 8 of 11 patients recruited for this preliminary study. False-positive PET was due to a tubular adenoma and a polyp with high-grade dysplasia, but no frank malignancy (Veit *et al*., 2006a). Nonetheless, since these lesions are potentially premalignant, they deserve clinical attention. Overall, PET alone is sensitive in identifying primary CRC, with a sensitivity of 95–100%, as shown by a few studies (Cohade *et al*., 2003). It should be borne in mind, however, that lower detection rates (with up to 40% false-negative PET exams) may be encountered in the mucinous subtype of CRC. There is a direct correlation between tumor cellularity and FDG uptake in these tumors, and an inverse relationship with the amount of mucin production (Berger *et al*., 2000).

One study on 44 patients has shown that multi-detector CT is comparable to FDG-PET in detecting nodal metastases. The number of distant metastases

in this group of patients was too small to perform statistical analysis on distant metastases; however, FDG-PET and CT appeared to be complementary (Furukawa *et al*., 2005). FDG-PET is more sensitive than sonography, CT or MRI in detecting liver metastases in patients with gastrointestinal malignancies. In a meta-analysis including 61 different studies with 3,187 patients, sensitivity estimates on a per-patient basis for nonhelical CT, helical CT, 1.5-T MRI, and FDG-PET were 60.2%, 64.7%, 75.8%, and 94.6%, respectively; and on a per-lesion basis, sensitivity estimates for nonhelical CT, helical CT, 1.0-T MR imaging, 1.5-T MR imaging, and FDG-PET were 52.3%, 63.8%, 66.1%, 64.4%, and 75.9%, respectively (Bipat *et al*., 2005). High-spatial-resolution mangafodipir trisodium-enhanced liver MRI and whole-body FDG-PET are comparable in the detection of patients with CRC liver metastases with a sensitivity of 96.6% and 93.3%, respectively. Mangafodipir trisodium-enhanced liver MRI appears to have an advantage in identification of subcentimeter liver metastases over FDG-PET (Sahani *et al*., 2005). It is possible that increasing scan duration (and thus higher count rates from small metastatic foci) as well as respiratory gating may increase the sensitivity of FDG-PET in detecting small liver metastases, below 1-cm in diameter.

Multiple studies have shown that PET/CT has an advantage over PET alone in identification of tumor sites (Metser *et al*., 2005). Correct staging of CRC was better for PET/CT than for PET alone (89% vs. 78%, respectively), with an increase in diagnostic confidence and a decrease in equivocal lesions requiring further work-up (Cohade *et al*., 2003). There is an advantage to fusion imaging for characterization of abdominal tumor sites as compared with side-by-side reading of PET and CT performed separately, especially for identification and characterization of small lymph nodes, lesions adjacent to mobile organs such as bowel, or lesions adjacent to the abdominal wall, such as peritoneal or omental deposits (Metser *et al*., 2005) (Figure 13.2). A large-scale prospective PET/CT study preferably with a multidetector CT scanner is needed to assess the accuracy of local staging of CRC with PET/CT, as compared with conventional

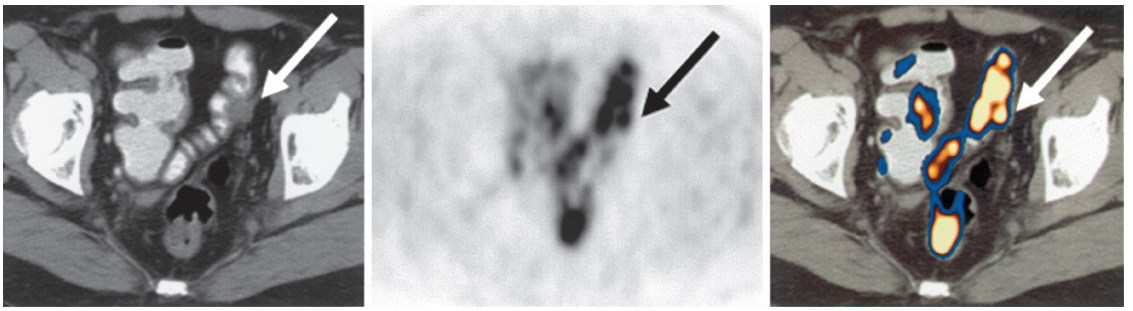

FIGURE 13.2. PET/CT performed 6 months after surgery for colon cancer (CT on left, PET in middle and fused PET/CT on right). Note extensive physiological uptake of FDG is noted on bowel, with no identifiable abnormality on PET. Subtle serosal metastatic deposit was missed on separate CT scan performed a few days earlier, but easy to confirm on fused PET/CT image (arrow)

multi-detector CT. Staging with fusion imaging may add the advantages of the high temporal and spatial resolution of state-of-the-art multi-detector CT for local staging, with the improved sensitivity and specificity of functional imaging.

ASSESSMENT OF RESPONSE TO THERAPY

Assessment of response to therapy in cancer patients is crucial to enable individualized tailoring of therapy. Patients demonstrating no response or progressive disease under therapy may require a change in therapeutic regimen. There are limitations to anatomic imaging modalities such as sonography, CT or MRI in assessing response to therapy. Morphological imaging modalities rely on change in size as a primary criterion for response to therapy. There is a relatively prolonged time for measurable tumor shrinkage to occur after cell death, making early prediction of response difficult. Furthermore, anatomic imaging modalities may also be limited in distinguishing viable residual tumor from residual necrotic and fibrotic tissue, when assessing patient after completion of therapy (Even-Sapir *et al*., 2004).

Positron emission tomography, which is based on assessment of metabolism, can detect response or lack of response at an earlier stage. For certain cancers such as lymphoma or metastatic breast cancer, studies have shown that sequential FDG-PET can predict response to treatment when a significant decrease in FDG uptake occurs even after one cycle of chemotherapy, whereas non-responding tumors show an increase, no change, or only a small decline in FDG uptake (Dose Schwarz *et al*., 2005). In these studies, an early response to treatment as shown by PET has generally correlated well with the ultimate clinical, radiographic, and pathological response recorded several weeks or a few months later. There are several factors that influence assessment of response to therapy by FDG-PET, including patient related factors (such as fluctuation in serum glucose levels between studies), and factors generated by therapy (such as an inflammatory response generated by radiotherapy). Nonetheless, resolution of FDG–uptake after chemoradiation for locally advanced rectal cancer appears to be the best predictor of survival in patients with locally advanced rectal cancer undergoing surgery with curative intent.

Neoadjuvant Chemoradiation Therapy

Neoadjuvant chemoradiation has been shown to decrease the frequency of local recurrences and may also improve long-term survival. Several studies have been performed on the performance of FDG-PET in assessing neoadjuvant therapy in these patients. In one prospective study including 21 patients with T3-T4 rectal cancers, a cutoff value of 36% in SUV reduction after neoadjuvant therapy differentiated responders from nonresponders with a sensitivity of 100% and specificity of 85.7%, as compared to surgery performed within 6–8 weeks after completion of therapy (Amthauer *et al*., 2004). Therefore, although PET lacks the spatial resolution to assess T-stage, it is accurate in assessing T-stage response. Transrectal sonography traditionally used to evaluate T-stage response to therapy was concordant with histopathological staging in only 41.2% of patients while overstaging ~ 53% of them. This may be explained

by radiation-induced inflammatory and/or desmoplastic reactions and necrosis which may cause over-estimation of tumor depth. These findings are in agreement with findings from previous studies, as well as studies utilizing MR imaging to assess response, showing an accuracy of 52% in re-evaluating T-stage of irradiated tumors (Chen *et al*., 2005).

Several pitfalls of FDG-based metabolic imaging should be taken into account when assessing response to chemoradiotherapy. If restaging is performed early after chemotherapy or radiation therapy false-negative PET results may be obtained. This is likely due to transient reversible decrease in glucose metabolism due to "stunning" of tumor cells. Conversely, radiation-induced inflammatory changes may occur up to 6 months after radiation therapy. Because FDG is not tumor-specific and uptake of FDG is seen in inflammatory tissue (especially tissue rich in activated macrophages), these inflammatory changes may show increased uptake of FDG, indistinguishable from residual tumor. Therefore, the accepted recommendation in the literature is to restage patients at an interval of at least 6–8 weeks from completion of radiation therapy. However, since patients undergoing neoadjuvant chemoradiotherapy for locally advanced rectal tumors are scheduled to undergo surgery within 6–8 weeks from completion of therapy, this may not be technically feasible. A more recent study has shown that false-positive results are not likely to occur if studies are performed 4 weeks from last radiation therapy (Amthauer *et al*., 2004), offering clinicians a two week interval to perform optimal metabolic response assessment to therapy before surgery.

Chemotherapy for Metastatic Colorectal Cancer

Response to chemotherapy for metastatic CRC has been addressed by several researchers. Findlay *et al*. (1996) assessed 18 patients with 27 metastatic liver lesions before, 1–2 weeks and 4–5 weeks after starting fluorouracil (5 FU)-based chemotherapy. They found no correlation between pretherapy level of FDG uptake as expressed by tumor to liver ratios and SUVs. Although response to therapy was associated with lower uptake of FDG 1–2 weeks and 4–5 weeks after starting chemotherapy, the 4–5 weeks tumor to liver ratio was able to discriminate response from nonresponse, both in a lesion-by-lesion basis and overall patients response assessment with sensitivity of 100% and specificities of 90%, and 75%, respectively. It is clear from these results, that timing of PET scan is crucial in assessing response to therapy. In a prospective study of 42 patients before liver resection for CRC hepatic metastases preoperative chemotherapy significantly decreased the sensitivity of FDG-PET in identifying tumor sites. Surgical specimens were also analyzed for hexokinase activity, with significantly lower hexokinase activity found in treated metastases, explaining the lower uptake of FDG and lower tumor detection rate in these patients (Akhurst *et al*., 2005). Therefore, FDG-PET scans must be read with full knowledge of recent therapy. Moreover, a negative FDG-PET study cannot reliably exclude residual viable neoplastic disease in patients recently treated with chemotherapy.

An additional approach for monitoring response to therapy is the kinetic approach, measuring FDG uptake over time, facilitating detection of more subtle

changes in tumor uptake of radiotracers than visual analysis or SUV measurements. Such measurements which normalize radiotracer concentration for injected activity and body weight are helpful in routine clinical practice, but harbor several limitations. Particularly, fluctuating glucose levels or change in body weight (due to reduced uptake of FDG in adipose tissue) between studies, differences in time between injections of radiotracer and imaging acquisitions, and inaccuracies in measured injected activity due to extravasation at the injection site may all potentially have bearing on serial SUV measurement. Compartmental models, which enable quantitative assessment of tracer kinetics, were introduced by Sokoloff *et al*. (1977) and used for *in vivo* analysis of several radiotracers. In these models, each compartment represents a tracer in a different space or in a different chemical composition. Tracers move through compartments and are bound by conservation of mass. The rate constants $K_{1–4}$ refer to the rate constants between compartments: K_1 and K_2 represent the transfer of FDG in and out of cells, respectively, by glucose transporter proteins; K_3 represents phosporylation of FDG by hexokinase and K_4 represents dephosphorylation by glucose-6-phosphatase.

Several reports on PET with kinetic modeling have shown that this methodology enables more accurate assessment of tumors. Specifically, higher influx rate constants (K_1) are measured for malignant lung lesions, as compared with inflammatory lung lesions, which show a relatively rapid washout of FDG (Gupta *et al*., 1998). A few studies have used kinetic modeling to assess tumor response to therapy. Combination of data from serial dynamic studies and SUV measurements on patients with metastatic CRC may predict response to therapy and long-term survival, with K_3 and K_4 of studies performed after first and third cycles of chemotherapy having the best statistical correlation with overall response (Dimitrakopoulou-Strauss *et al*., 2004). It should be borne in mind that metastatic disease in a single patient may show mixed response to systemic therapy, with response in certain sites and progression of disease in others. This may limit the use of complex dynamic imaging of a single or a few metastatic foci to predict overall response and prognosis.

Other Radiopharmaceuticals for Metastatic Colorectal Cancer

The most frequently used labeled cytostatic agent is ^{18}F-fluorouracil (FU). Because radiolabeled ^{18}F-FU is identical to FU, PET has been used to evaluate and study its distribution and metabolism. Patients with high ^{18}F-FU uptake values, indicating high uptake of the chemotherapeutic agent within tumor, were more likely to achieve at least stabilization of disease with FU (Moehler *et al*., 1998). This method potentially offers clinicians the opportunity to evaluate which patients are likely to have good response and improved survival before commencement of FU-based chemotherapy. There also appears to be correlation between uptake of ^{18}F-FU in liver metastases and their growth rate as measured by CT.

Other radiopharmaceuticals used for PET in CRC are based on assessment of cellular proliferation. Because deoxyribonucleic acid (DNA) synthesis is necessary for cellular proliferation occurring in the S phase of the cell cycle, labeled thymidine has been used to study cell growth.

Exogenous thymidine may enter a cell by facilitated diffusion, and is ultimately phosphorylated to a triphosphate prior to incorporation into DNA. A few positron emitters have been used to label thymidine for PET imaging; however, ^{18}FLT (3'-deoxy-3'-fluorothymidine) is a relatively stable thymidine analog, with the advantage of the relatively long half-life of ^{18}F (~ 110 min).

Once in a cell, ^{18}FLT is converted by thymidine kinase 1 (TK1) to a monophosphate (^{18}FLT-6-PO4) and trapped within the cell. High TK1 activity correlates with high proliferative rate of a cell, including CRC cells (Sakamoto *et al*., 1984). ^{18}FLT-PET correlates with cellular proliferation markers in both primary and metastatic CRC. However, in clinical use, the high background activity in the liver after injection of ^{18}FLT hampers the detection of metastatic lesions in the liver (34% detection rate, as compared with 97% for FDG). Extrahepatic metastases were detected fairly well, 92% of metastatic lesions identified on FDG-PET were also identified on ^{18}FLT-PET (Francis *et al*., 2003). Despite the lower sensitivity of ^{18}FLT for the detection of tumor sites in patients with CRC, although not proven to date, it may have a potential role in improving specificity and in the assessment of response to therapy.

Local Therapy for Metastases

Several hepatic-directed therapies are available for treatment of unresectable CRC liver metastases, including intraarterial chemotherapy, conformal radiation, ^{90}Y microsphere therapy, and ablative techniques. Local ablative techniques such as cryosurgery ablation (CSA) and radiofrequency ablation (RFA) are being used for the treatment of colorectal liver metastases, mainly as an adjunct to surgical resection of hepatic metastases, when complete tumor-free liver cannot be obtained by surgical resection alone. The success of this form of therapy relies on accurate monitoring of tumor destruction at the time of treatment and accurate detection of early local recurrence, because recurrence rates range between 2–55% for RFA (Rossi *et al*., 1996), with similar results for CSA. Early detection of local recurrence may offer the opportunity to reintervene by repeat ablation or surgery.

Sonography is used intraoperatively for the assessment of tumor destruction during the procedure, and sonography or CT can be used when performed percutaneously. For post-procedure assessment of tumor recurrence, CT and MRI are traditionally used. When comparing the performance of FDG-PET performed after ablative therapy to contrast-enhanced CT, PET appeared to be more accurate for detection of residual tumor, with an accuracy of 68% for PET and 47% for CT (Veit *et al*., 2006b). Although this study was a small preliminary study, the author's findings are supportive of other studies. When FDG-PET performed within 3 weeks of RFA, FDG-PET was able to detect residual tumor locally better than CT (Figure 13.3). A false-positive case was encountered in a patient that developed an abscess after RFA, which showed persistent FDG uptake. The negative predictive value of post-ablative FDG-PET was high, with none of the patients developing local recurrence during a mean follow-up period of 16 months. FDG-PET detected recurrent metastatic disease in the liver outside the ablated region or extra-hepatic

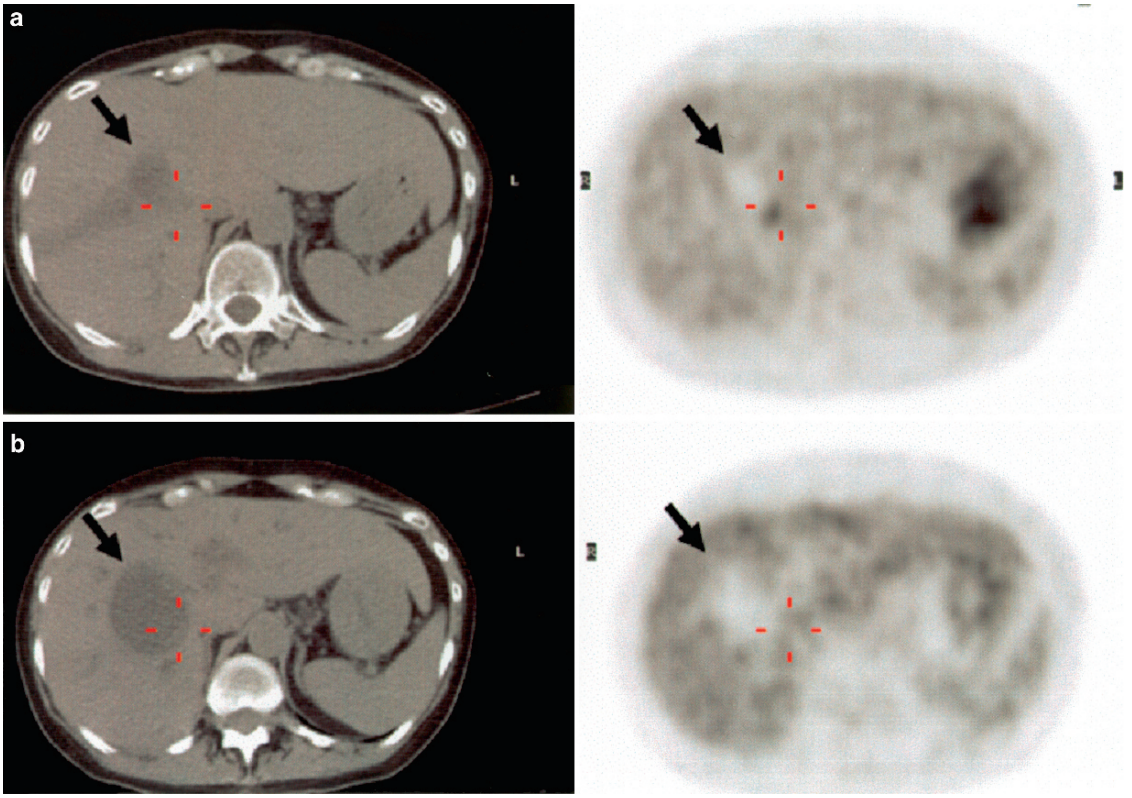

FIGURE 13.3. a: PET/CT performed 3 weeks after sonographically-guided radiofrequency ablation of a metastatic deposit in segment 8 of the liver. A subtle focus of increased FDG uuptake is noted along posteromedial aspect of necrotic lesion (arrow), indicating residual viable tumor (red cross). b: Repeated PET/CT after re-ablation shows more extensive necrosis, appearing photopenic on PET scan, without evidence of residual tumor

metastases earlier than CT (Langenhoff *et al*., 2002).

Intrahepatic arterial ^{90}Y-glass microsphere therapy is a form of brachytherapy, administered into the hepatic artery on angiography. Since hepatic metastases receive blood supply from the hepatic artery, whereas the liver parenchyma receives its blood supply primarily from the portal venous system, high radiation doses can be delivered to tumor with relative sparing of liver parenchyma. Preliminary reports on the efficacy of intrahepatic arterial ^{90}Y-glass microsphere therapy of unresectable CRC liver metastases show that tumor response rate, defined as 50% reduction in the product of the longest perpendicular diameters for measurable lesions, was 23–35%, with median survival of 13.5 months for patients with ≤25% tumor burden and good clinical performance status. Toxicities were minimal. FDG-PET can demonstrate response to this form of therapy both quantitatively and on visual inspection alone, by showing significant reduction in tumor metabolism. There appears to be a large discrepancy between anatomic response as assessed on CT, and

metabolic response, as assessed on PET after ^{90}Y-glass microsphere therapy (Wong *et al*., 2004).

IDENTIFICATION OF TUMOR RECURRENCE

Recurrence of CRC after surgery with curative intent is common, with reported relapse in more than a quarter of patients. In a series of 524 patients, anastomotic recurrence occurred earlier than distant metastases (mean of 16.2 months vs. 22.9 months, respectively). Anastomotic recurrence was significantly more frequent in patients with rectal than colon cancer, with > 20% recurrence rate. Aside from site of primary tumor, positive predictive factors for recurrence included stage (recurrent tumor occurred in almost half of patients with nodal metastases at diagnosis), invasion of contiguous organs, and presence of perforation. Age, gender, degree of differentiation, and mucinous subtype were not found to be predictive factors of recurrence. When re-resection is possible, up to 50% of patients may have long-term survival (Veit *et al*., 2006a).

Strict patient selection for re-resection is crucial to avoid unnecessary surgery and morbidity in patients in whom surgery would not be productive. Conventional imaging strategies have had limited success in patient stratification, with more than half of the patients who are thought suitable for curative surgery being found to have unresectable disease at surgery. Comparative studies between conventional imaging strategies and FDG-PET have shown that PET is more sensitive in detecting recurrent disease, 93% vs. 69% in a prospective blinded study on 115 patients (Valk *et al*., 1999), and more accurate than CT in overall restaging of patients. Including PET in the imaging strategies of patients with metachronous liver metastases from CRC is cost-effective (Lejeune *et al*., 2005), as shown by a few studies performed to date. On the basis of this study and previous data, 33.3% of patients with liver recurrence are assumed to be directed toward inappropriate surgery after CT, 19% after MRI, and 17.4% after FDG-PET. Similarly, 5.3%, 3.8%, and 1.3% of patients may be falsely upstaged after CT, MRI, and FDG-PET, respectively.

Fusion imaging appears crucial for correct identification of local recurrence and distant metastatic disease in patients with CRC, as several studies have shown significant benefit for PET/CT over PET alone, with higher sensitivity and markedly higher specificity (69% for PET and 92% for PET/CT). False-positive results are mostly due to inflammatory lesions (Votrubova *et al*., 2006). PET/CT also has a significant advantage over CT or PET alone in the evaluation of pelvic recurrence in patients after abdomino-perineal resection or anterior resection of rectal cancer. In another study with 81 suspected tumor sites in the pelvis, the sensitivity, specificity, and accuracy for differentiating malignant from benign FDG uptake in the pelvis were 98%, 96%, and 93% for PET/CT and 82%, 65%, and 74% for PET, respectively. Detection of masses is dependent on comparison with normal anatomy, which could be markedly distorted by radiation and/or surgery in the pelvic region. After abdominoperineal resection, there is often posterior and inferior displacement of pelvic organs into the vacant rectal fossa. Indeed, physiologic FDG uptake in displaced pelvic organs

was the most common reason for false-positive PET, with physiological uptake of FDG clearly sorted out on fused PET/CT. In addition, due to the ability of FDG-PET to reliably distinguish between fibrosis and viable tumor, PET/CT was able to discriminate between benign and malignant presacral abnormalities with a sensitivity and specificity of 100% and 96%, respectively (Even-Sapir *et al*., 2004).

After curative surgery, patient monitoring is performed to detect recurrence as early as possible in order to improve patients' survival. Patients undergo serial clinical and imaging workup, a search for metachronous cancers by endoscopy, as well as monitoring of serum tumor markers, most notably carcinoembryonic antigen (CEA) levels, which may help detect asymptomatic recurrences. This antigen is a glycoprotein that was initially associated only with colorectal cancer and embryonic gut tissue. However, it is an imperfect tumor marker, with elevated levels shown in many other malignant diseases, as well as various benign conditions (including inflammatory conditions of the bowel and lung, pancreatitis, heavy smoking and alcoholic liver disease). In patients with CRC it is elevated only when tumor cells have penetrated through the bowel wall (i.e., at least Stage B2). It may be markedly elevated when liver involvement exists, but may not reflect true extent of disease in patients with bulky extrahepatic, intra-abdominal recurrence, or in poorly differentiated tumors. In ~ 90% of patients with elevated CEA levels after surgery, tumor recurrence exists; however, only 12–60% of these patients have resectable disease (Cohen *et al*., 1993). Identifying tumor recurrence in patients with rising tumor markers with normal colonoscopy and normal or equivocal CT is a clinical challenge. In recent years, FDG-PET has been utilized for this purpose and found to be sensitive in detecting recurrence, with a positive predictive rate of 89% on a patient-based analysis in a few studies (Flamen *et al*., 2001). In addition, there appears to be correlation between tumor load as assessed on FDG-PET and CEA levels. In one study, bulk of disease was measured by performing an isocontour plot of tumor masses at an SUV of 2.5, termed "PET volume", with a linear correlation found with serum CEA levels (Choi *et al*., 2005).

POSITRON EMISSION TOMOGRAPHY AND COLORECTAL CANCER: FUTURE DIRECTIONS

Molecular imaging modalities such as SPECT and PET use radiolabeled molecules to image molecular interactions *in vivo*, in animal models (with unique micro-PET systems) or human subjects. Due to its proven accuracy in predicting response to therapy at an early stage, radiopharmaceutical companies testing new drugs may incorporate FDG-PET into clinical trials. Metabolic response may prove to be more sensitive and specific than the commonly used RECIST criteria of anatomic imaging modalities.

Positron emission tomography can be used to quantitatively assess the distribution of positron emitters that can be incorporated into receptor radioligands, enabling reliable imaging status *in vivo* of receptors. Metabolic imaging techniques may help develop new molecules to image function, or modify the function of targeted

cells when given in larger amounts. For example, a recently developed positron emitting radiopharmaceutical for neuroendocrine tumors, ^{68}Ga-DOTATOC, results in high tumor to nontumor contrast and low kidney accumulation and yields higher detection rates as compared with ^{111}In-octreotide scintigraphy (Hofmann *et al*., 2001). This may prove to have therapeutic implications. ^{124}I-labeled engineered anti-CEA minibodies and diabodies allow high-contrast, antigen-specific small-animal PET imaging of xenografts in athymic mice, and a promising new genre of tumor-specific probes for PET imaging of tumors (Sundaresan *et al*., 2003). Gene-based therapy is a promising therapeutic approach for many types of cancers. However, to date clinical success has been limited. This may be due to difficulties in monitoring gene expression at the targeted site *in vivo*. Molecular imaging may enable real-time assessment of the therapeutic process and the refinement of treatment protocols, and has been successfully utilized in the non-invasive assessment of gene transfer and gene therapy in humans (Iyer *et al*., 2005).

In conclusion, since its incorporation into routine use in clinical medicine several years ago, FDG-PET has improved the management of patients with CRC. Its major contribution so far has been in early and accurate assessment of response to therapy, as well as enabling more appropriate patient selection for metastasectomy, as compared with anatomic imaging modalities. Fusion imaging with state of the art PET/CT scanners has for the most part overcome PET's main limitation of low spatial resolution and lack of anatomic landmarks, increasing sensitivity and specificity of staging and restaging of patients with CRC. Molecular imaging in general, and specifically PET, will undoubtedly have a significant role in the development, testing, and clinical implementation of new oncological therapeutic agents in the future.

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14
Prognostic Significance of Protein Markers in Colorectal Cancer Stratified by Mismatch Repair Status

Inti Zlobec and Alessandro Lugli

INTRODUCTION

Colorectal cancer CRC is a leading cause of cancer-related mortality in North America and Western Europe. Although early stages of the disease are linked to excellent post-operative prognosis and a cure rate of 80–95%, patients with invasive cancers and lymph node metastasis have a 5-year survival rate of 25–60% (Compton and Greene, 2004). Targeted-therapy based on the individual gene or protein expression profile of the tumor is expected to improve outcome and response to chemotherapy or radiotherapy by assisting in the selection of candidate patients for specific treatment protocols (Ghadimi *et al*., 2005).

Colorectal cancers are classified at the molecular level into two main groups. The majority of CRCs occur sporadically 80–90% and arise through the "tumor suppressor pathway" involving sequential mutations and loss of heterozygosity in several tumor suppressor genes such as adenomatous polyposis coli (APC), deleted in colon cancer DCC, p53, and KRAS (Weitz *et al*., 2005). Approximately 15% of CRCs develop as a consequence of the "mutator pathway" characterized by inactivation of the DNA mismatch repair, MMR machinery leading to microsatellite instability (MSI), and immunohistochemical negativity for proteins MLH1, MSH2, and MSH6 (Jass, 2004a).

Colorectal cancer with MSI is shown to have a better prognosis than stage-matched microsatellite stable CRC (Jass, 2004a). MSI in hereditary and sporadic cancer occurs through two different mechanisms. In hereditary non-polyposis coli cancer (HNPCC) the cause for MMR deficiency is a germline mutation in one of the MMR enzymes (Jass, 2004a). Sporadic CRC with MSI is often caused by loss of *MLH1* expression due to promoter hypermethylation and possesses mutations including those of BRAF, transforming growth factor β receptor II (TGFβRII), insulin growth factor 2 receptor (IGF2R), and BAX rather than those of APC, KRAS or p53 seen in the tumor suppressor pathway (Fujiwara *et al*., 1998; Jass, 2004b).

Several signal transduction pathways have been implicated in the progression and metastasis of CRC including WNT, Ras/Raf/MEK/ERK, AKT/PI3 kinase and TGF-β signaling pathways (Weinberg, 2007). The immunohistochemical detection of proteins involved in these and other mechanisms has yielded important

information as to their value as prognostic or predictive markers in CRC.

TISSUE MICROARRAY TECHNOLOGY

The tissue microarray TMA is an unparalleled, cost-effective resource for studying the protein expression of tumor markers (Goethals *et al*., 2006; Sauter *et al*., 2003). TMAs allow the investigation of protein expression in hundreds of CRCs from patients at different stages of disease and with various lengths of follow-up (Figure 14.1). Traditional methods of molecular pathology render nearly impossible the analysis of such a huge number of histologically well-characterized tumors. Not only would such an ambitious project on whole tissue specimens generate a massive workload for the laboratories involved but also tissues would be rapidly depleted. Moreover, TMA technology has several additional benefits over the use of regular slides. First, several studies have shown previously well established associations between molecular features and clinico-pathological endpoints

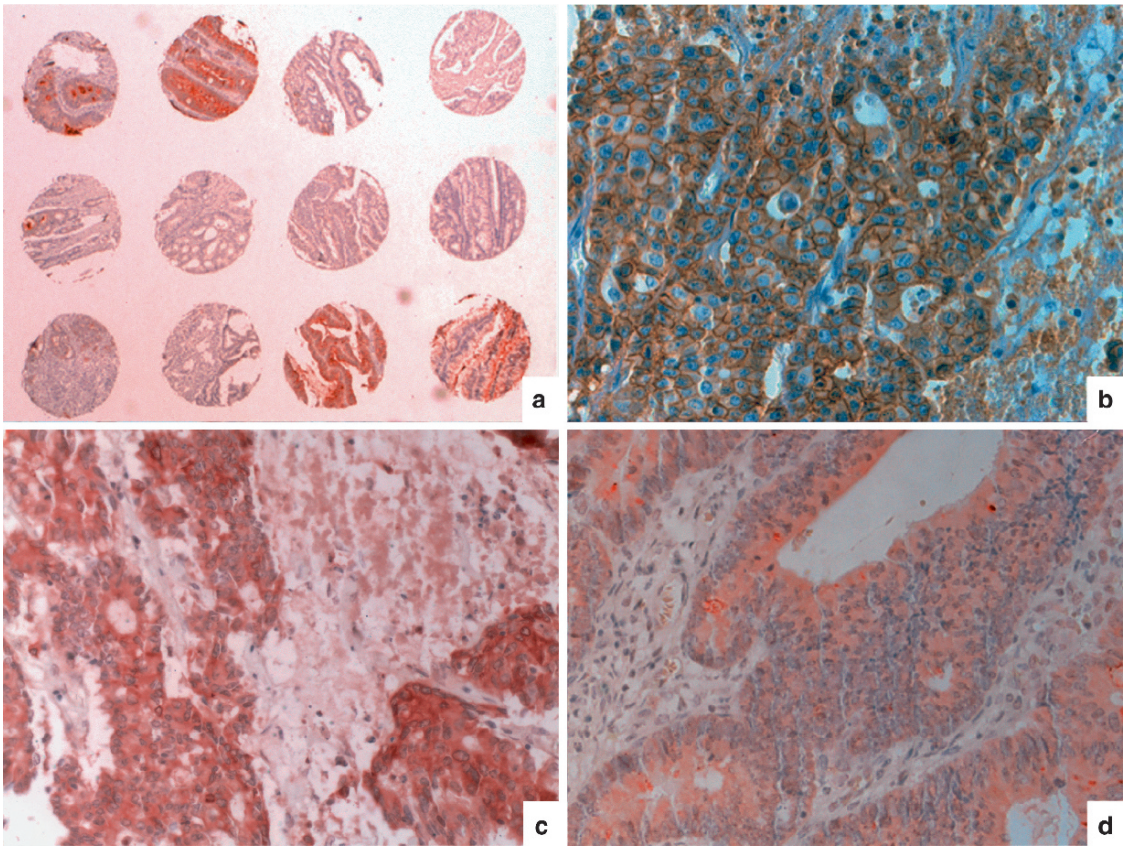

FIGURE 14.1. TMA showing MUC1 expression in colorectal cancer (2.5x) (a). Membraneous expression of EGFR (40x) (b), cytoplasmic expression of RKIP (40x) (c) and APAF-1 (40x) (d) in moderately differentiated adenocarcinomas of the colon

in TMAs (Barlund *et al*., 2000). Second, the greater objectivity of the staining interpretation on one small tissue fragment and the high level of standardization are probable factors that may compensate for the disadvantage of the small sample size (Sauter *et al*., 2003). Third, it is often impossible to include several punches per tumor using small tissue specimens as donor tissue and, therefore, it is better to collect data from large series of tumors to determine the prognostic significance of protein expression (Goethals *et al*., 2006). Finally, the large sample size of the TMA allows for more reliable and reproducible statistical modeling.

MANAGEMENT OF TISSUE MICROARRAY DATASETS

A possible approach to study a large number of unselected CRCs using the TMA is to stratify all cases by MMR status. In several studies, CRCs were classified as likely sporadic and microsatellite stable expressing *MLH1*, *MSH2* and *MSH6*, likely sporadic *MSI-H MLH1*-deficient and > 55 years of age and probable HNPCC loss of MSH2 and/or MSH6 or loss of *MLH1* at age 55 years or younger. These immunohistochemical groupings showed a good fit with the known clinico-pathological features associated with these subsets of CRC. In particular, the *MLH1*-negative group was associated with advanced age, predilection for females and proximal colon, large tumor size, and poor differentiation. The presumed HNPCC group was young, showed no gender difference, and there was a predilection for the proximal colon as compared with the MMR-proficient group.

SCORING SYSTEMS AND STATISTICAL ANALYSIS

Scoring systems for tumor markers detected by IHC in CRC are typically based on some measure of the number of positive tumor cells and often combined with a degree of staining intensity. However, it has been demonstrated using an anti-EGFR antibody in head and neck cancer, non-small cell lung carcinomas and colorectal adenocarcinoma that the degree of staining intensity varied by tumor type, was partially influenced by the choice of fixatives and was inversely correlated with storage time of the unstained tissue sections (Atkins *et al*., 2004). These factors, in addition to the variation in IHC protocols, inevitably contribute to the subjective nature of staining intensity. Contradictory results from different reports on the same tumor markers may be partially explained by this subjective assessment of immunoreactivity.

An additional obstacle faced by researchers and pathologists involved with IHC is the determination of the extent of tumor positivity for a given marker which is clinically and biologically relevant. This is often assessed using a pre-determined cut-off score which, particularly for novel tumor markers, is often set arbitrarily and varies between different reports. One possible alternative to these standard evaluation methods is the determination of immunoreactivity based on the semi-quantitative assessment of the percent positivity of positive tumor cells. The reproducibility of this scoring system has recently been demonstrated among pathologists for several proteins, namely EGFR, VEGF, p53, Bcl-2 and APAF-1 in tumor biopsies and TMA punches (Zlobec *et al*., 2006a, 2007a, b).

This descriptive, semi-quantitative scoring system has several advantages over methods based on pre-determined cut-off scores (Lugli *et al*., 2006a). First, this scoring system allows a more thorough assessment of the predictive or prognostic significance of tumor markers by evaluating the entire range of protein expression levels from 0% to 100%. The correlations between various proteins can be assessed. It has been recently shown using this approach that the percentage of pERK positive tumor cells is strongly associated with increases in RHAMM expression supporting the hypothesis of a RHAMM-MAP kinase interaction in MMR-proficient CRC (Lugli *et al*., 2006a). Most importantly, by quantifying protein expression at the outset, more relevant cut-off scores for tumor positivity can be established using statistical approaches such as Receiver Operating Characteristic (ROC) curve analysis (Zlobec *et al*., 2007c).

ROC curves are commonly used in many areas of medical research and in clinical oncology to evaluate and compare the sensitivity and specificity of the diagnosis. In addition, they can be used to identify the threshold value above which a test result should be considered positive for some outcomes (Hanley, 1989). The semi-quantitative assessment of scores therefore permits ROC curve analysis to be applied similarly to evaluate IHC protein expression and to select more appropriate threshold values for tumor positivity.

The ROC curve is generated by evaluating the sensitivity and specificity of each protein expression score for the endpoint under investigation. In Figure 14.2, the ROC curves for the novel tumor marker RHAMM are illustrated for several different clinico-pathological features including T stage, N stage, tumor grade, the presence of vascular invasion and survival (Zlobec *et al*., 2007c). The protein expression score leading to the greatest sensitivity and specificity for the outcome can be identified from the ROC curve. The point minimizing the trade-off between sensitivity and specificity leads to the greatest overall number of correctly classified tumors with or without the clinico-pathological features. One method to select the optimal cut-off score is to choose the point on the curve with the shortest distance to the coordinate 0, 1 which corresponds in theory to the point with the maximum sensitivity and specificity for the outcome. From Figure 14.2, the cut-off scores for all features are easily obtained and include 100% for T stage, N stage, tumor grade and vascular invasion < 100% RHAMM staining versus 100% expression and 90% for survival ≤90% staining, versus > 90% staining.

In addition to these benefits, the semi-quantitative scoring method avoids an often complex and interpretative composite scoring system based on the intensity of staining. One such method includes a four-tier scoring of the intensity of staining 0, 1+, 2+, and 3+ coupled to either the mean percentage of positive tumor cells or to a categorical measure of the percentage of positive tumor cells, for example, 1–10%, 10–50%, and > 50%. A graded scoring system has also been used where the percentage of positive tumor cells is categorized 0 = no positivity, 1 = 1–25%, 2 = 25–50%, 3 = > 50%, multiplied by the degree of intensity 0, 1, 2, and 3 to obtain a score which is then dichotomized into "low" or "high" expression low = score < 6 and high ≥ 6. Others have reported only the degree of staining intensity regardless of the proportion of immunoreactivity or considered only staining intensities of 2+ or 3+ as positive for protein expression.

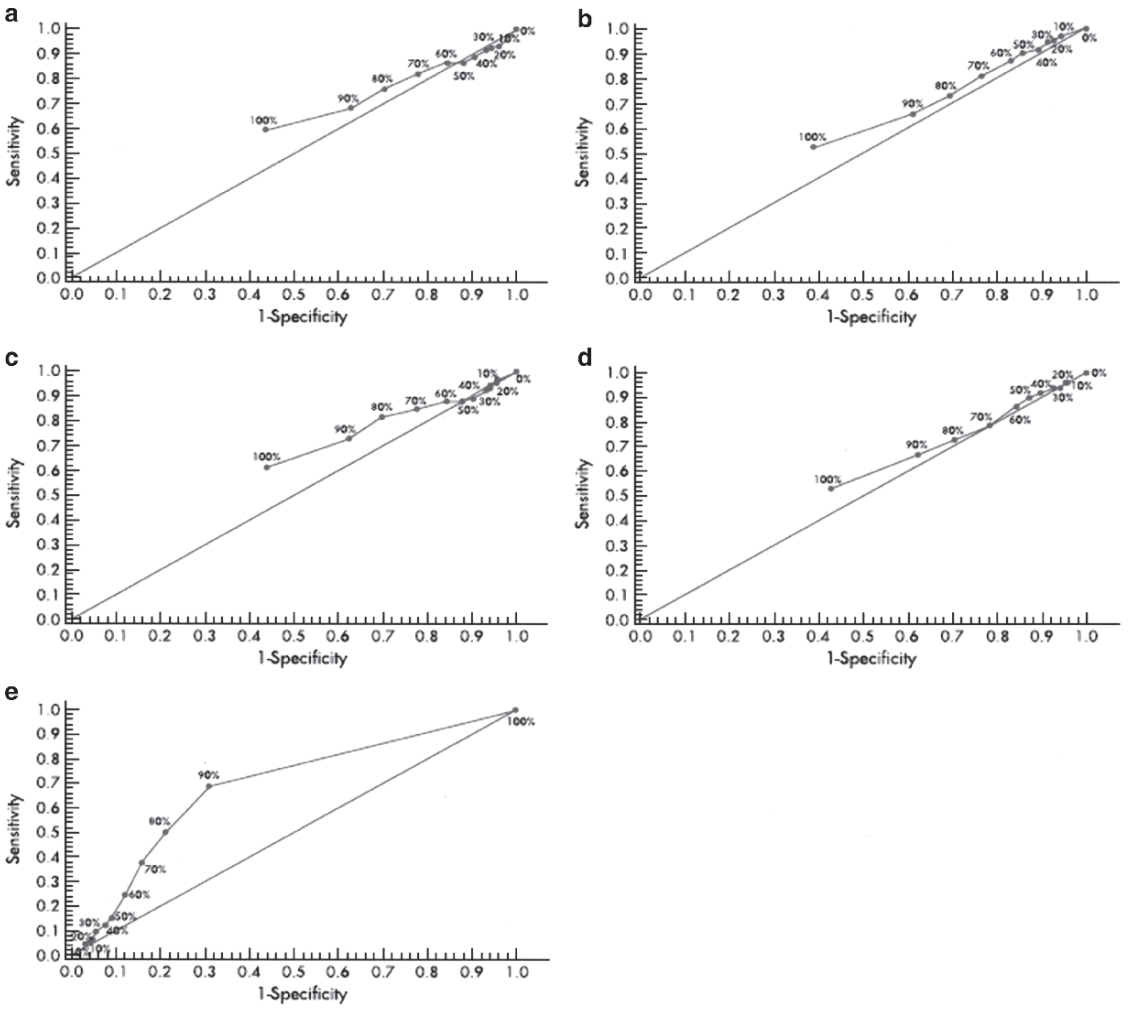

FIGURE 14.2. Receiver operating characteristic ROC curves for the novel tumor marker RHAMM and several important clinico-pathological features. ROC curves can be used to select cut-off scores for tumor marker positivity. By determining the protein expression score with the shortest distance to the point (0.0, 1.0), the cut-off score with the greatest sensitivity and specificity for the outcome can be selected. The 100% cut-off score leads to the best classification of patients with (a) early T1 + T2 versus late T3 + T4 pT stage, (b) no lymph node N0 versus any lymph node > N0 involvement, (c) low G1 + G2 versus high G3 tumor grade, and (d) absence versus presence of vascular invasion. (e) The 90% cut-off score is best to discriminate between patients who have died from colorectal cancer or were alive/censored at 5-year follow-up time

PROGNOSTIC FACTORS IN COLORECTAL CANCER STRATIFIED BY MISMATCH REPAIR STATUS

Recently, the role of several molecular markers in tumor progression and survival has been elucidated using IHC and the TMA approach. Dysregulation of the MAPK pathway was implicated in the mechanism of tumor budding in MMR-proficient CRC, while activation of the PI3K/AKT pathway appeared to be associated with the early stages of *MLH1*-negative CRC

progression (Lugli *et al*., 2006b). Mucins were determined to have prognostic significance in sporadic *MLH1*-negative CRC but not in HNPCC. Loss of MUC2 expression was found to have adverse prognostic significance in both MMR-proficient and *MLH1*-negative CRC, while expression of MUC1 was involved in tumor progression in MMR-proficient cases (Lugli *et al*., 2007a). A number of proteins implicated in the Wnt signaling pathway were investigated. It was found that increasing nuclear β-catenin expression and loss of membranous E-cadherin were independent, adverse prognostic factors in MMR-proficient and *MLH1*-negative CRC (Lugli *et al*., 2007b). The prognostic significance of RHAMM, a novel tumor marker previously unexplored in CRC was found to have strong, independent adverse prognostic value in MMR-proficient CRC and presumed Lynch syndrome HNPCC (Lugli *et al*., 2006a).

Several novel findings were obtained by implementing ROC curve analysis with the TMA to determine cut-off scores for tumor positivity. Loss of cytoplasmic Mst1 expression was significantly linked to tumor progression in both MMR-proficient and MMR-deficient cases, suggesting a tumor suppressing role for this protein in human CRC (Minoo *et al*., 2007b). Loss of cytoplasmic RKIP expression was not only found to contribute significantly to tumor progression but also was found to have independent prognostic information in *MLH1*-negative CRC and could have a potential role in predicting distant metastasis (Minoo *et al*., 2007a).The antiapoptotic marker APAF-1 emerged as a marker of tumor progression in MMR-proficient CRC and an independent adverse prognostic factor in *MLH1*-negative CRC (Zlobec *et al*., 2007a). Moreover, the loss of APAF-1 and E-cadherin within the main tumor body of CRCs was identified as an independent predictor of budding (Baker *et al*., 2006). EGFR predicted response to preoperative radiotherapy and was an independent adverse prognostic factor in CRC (Zlobec *et al*., 2007b). Moreover, by studying the TGFβ signaling pathway, insensitivity to TGFβ and increased TGFβ secretion were identified as independent predictors of high tumor infiltrating lymphocyte TIL counts, suggesting an important role for TGFβ signaling in the recruitment and retention of TILs within CRC epithelium (Baker *et al*., 2006).

USE OF MULTIMARKER PHENOTYPES

Prognostic markers in CRC are typically studied individually. However, most proteins do not act in isolation but rather within networks, or pathways with other proteins whose influence may confound the effects seen with univariate analysis. Multivariate analysis takes into account the relationships between different proteins, thus modeling with more accuracy the effect of each marker on, for example, survival time. Therefore, the evaluation of multimarker phenotypes should result in a better understanding of the role of each protein on prognosis in the context of the pathway to which it may belong.

This approach has been used recently with the TMA in MMR-proficient CRC to study proteins involved in cell-cycle arrest (Tornillo *et al*., 2007). Tumor markers p21, p53, p27 and bcl-2 were combined into a composite multimarker triplet score resulting in the following: p21/p27/bcl2,

p21/p27/p53, p21/bcl2/p53 and p27/bcl2/p53. An analysis of tumors positive for all three markers in the triplet demonstrated a significant difference in survival time between the first two groupings.

In the p21/p27/p53 triplet, all eight positivity and negativity combinations showed a significant association with prognosis. However, these associations were found to match the results obtained when analyzing p27 alone. It was, therefore, hypothesized that p27 may have a dominant role in determining patient survival. In particular, p27 loss may be a helpful marker in patients with node-negative pT3 CRC to identify those with a particularly unfavorable prognosis. The eight combinations of the p21/p27/bcl2 triplet were also correlated with survival time. Interestingly, p27− and p27−/p21+/bcl2+ patients had a worse prognosis in contrast to p27+ or p27−/bcl2+/p21+ patients who showed a better prognosis. A similar observation was made in pT2 and pT3N0 tumors.

DISCUSSION

RAS Signaling

pERK

The MAPK pathway is thought to be important for cellular growth, development and differentiation (Sebolt-Leopold, 2000). The downstream molecule ERK is activated by a cascade of phosphorylation events downstream from the ras proto-oncogene (Sebolt-Leopold, 2000). Recent studies have shown that ERK interacts with the wnt signaling pathway by inactivating GSK3β, an essential component of the GSK3B-APC-axin complex important in the process of β-catenin degradation (Ding *et al*., 2005).

In MMR-proficient CRCs, pERK was found to be associated with tumor budding which is supported by several reports (Lugli *et al*., 2006b). First, MEK inhibition in colon tumor models is shown to decrease invasiveness as well as inhibition of cell motility (Sebolt-Leopold, 2000). Second, KRAS and APC are mutated at particularly low frequency in the subset of sporadic CRCs showing high-level DNA microsatellite instability MSI-H, whereas they are identified in approximately 35% KRAS and 60% APC of unselected primary CRCs (Fujiwara *et al*., 1998; Jass *et al*., 2002b). Third, dysregulation of the wnt signaling pathway has been previously shown to be more likely associated with tumor budding in MLH1-expressing cancers rather than in CRCs with *MLH1*-loss (Young *et al*., 2001).

Raf-1 Kinase Inhibitor Protein RKIP

The Raf-1 kinase inhibitor protein has been implicated in the Ras/Raf/MEK/ERK pathway signaling, and is currently the only known cellular inhibitor of Raf-1 kinase (Theroux *et al*., 2007). Loss of cytoplasmic RKIP expression has been associated with the presence of distant metastasis, lymph node involvement, vascular invasion and worse survival in CRC (Minoo *et al*., 2007a). Methylation in the promoter region of *RKIP* has been reported in normal colon mucosa in patients with hyperplastic polyposis (Minoo *et al*., 2006) but not in MMR proficient and MMR deficient CRCs. Hence, other mechanisms, e.g., mutation, loss of heterozygosity may be responsible for downregulation of RKIP expression in CRC.

Epidermal Growth Factor Receptor EGFR

Epidermal growth factor receptor belongs to the ErbB tyrosine-kinase receptor family

which includes four proteins encoded by the c-erbB proto-oncogene, namely ErbB1 (EGFR), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4). Ligand binding produces dimerization of the receptor and activation of intrinsic protein tyrosine kinase activity leading to the transduction of signaling pathways involved in proliferation, cell division and differentiation. The MAP kinase and AKT signaling pathways have been found to mediate intracellular EGFR signaling.

Recently, ROC curves were applied to the IHC assessment of EGFR in MMR-proficient CRC (Zlobec *et al*., 2007b). A TMA of 1,197 tumors was randomized into two sub-groups, the first to establish an appropriate cut-off score for EGFR positivity, the second to determine associations of EGFR with several clinico-pathological features, T stage, N stage, tumor grade, vascular invasion, and 10-year survival. EGFR was scored semi-quantitatively by three independent pathologists and the scoring method was found to be reproducible. ROC curve analysis was performed on the average of the three scores and for each clinical endpoint the cut-off score for EGFR was ≥75%.

Epidermal growth factor receptor over-expression in MMR-proficient CRC was not associated with T stage, N stage, tumor grade or vascular invasion (Zlobec *et al*., 2007b). These results are supported by similar findings by other groups that have shown no relationship between EGFR over-expression and disease evolution (Goldstein and Armin, 2001). However, patients with EGFR over-expressing tumors demonstrated a significantly worse prognosis 36.5 months 20.0–65.0 than those without over-expression 82.0 months 66.0–96.0. Moreover, EGFR was found to predict worse survival in a multivariate analysis independently of known adverse prognostic factors including T stage, N stage, and vascular invasion. These results indicate that EGFR could be used as a prognostic marker in addition to pathological staging.

pAKT Signaling

pAKT

AKT is emerging as a central player in tumorigenesis (Testa and Bellacosa, 2001). The cancer hallmarks in which AKT plays a role are acquired growth signal autonomy, insensitivity to antigrowth signals, inhibition of programmed cell death, unlimited replication potential, sustained angiogenesis, tissue invasion and metastasis (Testa and Bellacosa, 2001). AKT is known to be expressed in normal colon while strong AKT immunoreactivity is also observed in both, colorectal adenomas and carcinomas. Cytoplasmic pAKT overexpression was demonstrated in early T stage and early N stage in *MLH1*-negative CRC (Lugli *et al*., 2006b). This supports the notion that activation of AKT is an early event in the development of colon cancer.

Cell Cycle Arrest Proteins

The role of p53 as "gatekeeper" of the cell cycle is well known (Vogelstein *et al*., 1988). p53 can induce apoptosis and can also impose a permanent block on cell division. It is therefore not surprising that loss of p53 could lead to poor outcome in cancer patients. However, the current literature on CRC and p53 are contrasting. Possible reasons could be the substantial methodologic heterogeneity of the published studies or simply the fact that a

"universal prognostic marker" does not exist. This observation encourages the use of multimarker phenotypes, as discussed in the previous section, along with clinicopathological parameters to determine CRC subgroups having different prognoses.

p27 plays an important role in the control of cell cycle, by "braking" the cyclin/cyclin dependent kinase effect together with other kinase inhibitors p21 and p57 (Chetty, 2003). Decreased expression of p27 has previously been associated with poor prognosis in CRC and additionally has led to the identification of a subset of patients with worse prognosis in a series of 97 early node-negative rectal cancer (Chetty, 2003) Furthermore, there is evidence to suggest that loss of p27 could be involved in the development of metachronous metastases and tumor growth in an environment of altered intercellular adhesion or extracellular matrix (Kane *et al*., 1997). Multivariate analysis indicated that of the clinico-pathological features and cell-cycle markers studied, only p53 positivity, pT, and pN were independent prognostic factors in MMR-proficient CRCs (Tornillo *et al*., 2007).

Wnt Signaling

β-Catenin and APC

The Wnt signaling pathway was recently investigated using a TMA of CRCs stratified by MMR status (Lugli *et al*., 2007b). Membranous β-catenin expression was associated with early T stage, early N stage, absence of vascular invasion and better survival in MMR-proficient CRC. In contrast, nuclear β-catenin expression was associated with higher N stage, presence of vascular invasion and worse survival in both univariate and multivariate analysis with known prognostic factors. These results are in line with the concept that wnt pathway dysregulation is accompanied by translocation of β-catenin from its normal membranous location to the cell nucleus. In addition, loss of cytoplasmic APC is associated with loss of membranous and increased cytoplasmic β-catenin expression in MMR-proficient CRCs.

A significant correlation between nuclear β-catenin expression and survival rate was found in CRC patients, while Cheah *et al*. (2002) observed that nuclear β-catenin expression was significantly related to higher mortality rates in 111 CRC patients. Whether increased nuclear β-catenin signaling is essential for the development of CRC is uncertain as at least 20% of CRCs show no nuclear staining in immunohistochemical analyses (Hao *et al*., 2001).

E-Cadherin

E-cadherin is a cell adhesion molecule that interacts with the wnt signaling pathway (Ilyas *et al*., 1997). It co-operates with α- and β-catenin as a functional unit termed the E-cadherin-catenin unit ECCU (Ilyas *et al*., 1997). E-cadherin is known to be decreased in invasive CRC (Bravou *et al*., 2006). Loss of membranous E-cadherin has been associated with higher T stage, higher N stage, and with the presence of vascular invasion in MMR-proficient CRC and with higher N stage and worse survival in MLH1-negative CRC (Lugli *et al*., 2007b). E-cadherin was found to have independent prognostic value in both CRC sub-groups.

In MMR-proficient and MLH1-negative CRC, an association between loss of membranous E-cadherin and loss of membranous β-catenin expression was described (Lugli *et al*., 2007b). This accurately

reflects the known biological interaction between E-cadherin and β-catenin. However, the absence of association between APC and β-catenin in MLH1-negative CRC indicates that activation of the wnt signaling pathway leading to accumulation of nuclear β-catenin may play an important role in tumor progression in MMR-proficient, but not in MLH1-negative CRC. In the latter group, loss of membranous E-cadherin seems to be an adverse prognostic factor independent of wnt signaling pathway activation. Since only a low incidence of E-cadherin mutations in CRCs has been observed, it is hypothesized that hypermethylation of the E-cadherin promoter may be the cause of decreased E-cadherin protein expression in CRC. This mechanism could be implicated in tumor progression in MLH1-negative CRC.

Tumor Infiltrating Lymphocytes (TILs) and Transforming Grwoth Factor-β (TGF-β) Signaling

Anti-tumor immune responses may be one of the most important weapons in the arsenal against cancer. A recent CRC study by Baker *et al*. (2007) which stratified cases according to MMR status confirmed that TILs are indeed an important favourable clinical and prognostic indicator but that these benefits may be confined mainly to the MMR proficient subset. These results supported and expanded upon those from a previous study which implicated disruptions to TGFβ signaling as one mechanism responsible for increased TIL presence (Baker *et al*., 2006). Increased Smad4 and Ki-67 were identified as predictors of elevated TILs. Additionally, increased TGFβ secretion emerged as a predictor of TILs in MMR deficient CRCs (Baker *et al*., 2007). The combination of increased TGFβ secretion and decreased tumor TGFβ sensitivity appears to be linked to elevated TILs in MMR proficient CRCs.

Evidence remains inconclusive over the exact role played by TILs in predicting CRC prognosis. It is likely that much of this controversy arises from inconsistent definitions of the term TILs, some studies including and some excluding stromal lymphocytes in this category, as well as from the lack of segregation of cases along MMR lines.

The normally high prevalence of TILs in MMR deficient cancers suggests that this feature may be inherent in the biology of the tumor rather than being reflective of an active immune response. The innateness of TILs to MMR deficient cancers is consistent with the theory of tumor immunoediting, which dictates that tumors reaching the stage of clinical detection have been antigenically shaped by the initial immune responses mounted against them to a point where they are no longer recognized as foreign to the body (Robinson *et al*., 2001). In MMR proficient cancers, which arise in a much more immunologically sparse environment, the tumors are less likely to have antigenically adapted and thus may be more sensitive to late stage immune attacks. Thus, even though mutator phenotype cancers are expected to produce a greater number of immunologically stimulating tumor specific antigens, the elevated levels of these coupled with the lack of appropriate co-stimulatory molecules on the tumor cells may generate a microenvironment which leads to a state of TIL anergy, thereby pre-empting any beneficial effect on survival.

The majority of MMR deficient CRCs possess inactivating mutations in TGFβRII

that render them insensitive to signaling by TGFβ (Baker *et al*., 2006; Jass *et al*., 2002a). A major negative autoregulatory feedback pathway is thus inactivated such that TGFβ no longer negatively regulates its own synthesis. While Baker *et al*. (2006) did not find differences between mean levels of TGFβ between MMR subgroups, univariate analysis revealed that increased TGFβ secretion was an important predictor of high TIL infiltration in MMR deficient CRCs. This was supported by the finding that increased proliferation and Smad4 expression (both of which are known to be decreased by TGFβ) emerged as important predictors of intraepithelial lymphocytic infiltration.

Potential Novel Protein Markers in CRC Receptor for Hyaluronic Acid Mediated Motility RHAMM

The receptor for hyaluronic acid mediated motility appears to be involved in cell motility and signaling as well as oncogenic events (Turley *et al*., 2002). Evidence suggests that RHAMM influences tumor progression and metastasis (Abetamann *et al*., 1996). In MMR-proficient CRC, IHC expression of RHAMM was correlated with higher N stage and worse survival in a univariate analysis and was an independent adverse prognostic factor in a multivariate analysis (Lugli *et al*., 2006a). In presumed HNPCC, RHAMM expression was associated with worse survival in both univariate and multivariate analyses. These findings support results obtained in a recent study using RT-PCR on tissue specimens of patients with CRC in which RHAMM mRNA levels were higher in tumor tissue when compared to adjacent normal tissue (Line *et al*., 2002).

Mammalian Sterile20-Like Kinase (MST1)

Mst1 was originally identified as a stress-activated protein participating in a wide range of apoptotic responses (Creasy and Chernoff, 1995). Despite extensive studies, the mechanisms and targets through which Mst1 regulates apoptosis are not well understood. Mst1 phosphorylation results in caspase activation and apoptosis both upstream and downstream of caspases (Glantschnig *et al*., 2002). Under resting conditions, Mst1 is localized predominantly in the cytoplasm but cleavage by caspases or phosphorylation lead to Mst1 translocation to the nucleus resulting in chromatin condensation and DNA fragmentation (Glantschnig *et al*., 2002).

In MMR-proficient CRC, loss of Mst1 has recently been correlated with higher T and N stages and worse survival (Minoo *et al*., 2007b). These results are consistent with Mst1 function in induction of apoptosis and tumor suppression. In MMR-deficient CRC, loss of cytoplasmic Mst1 was associated with worse survival. This CRC subtype is characterized by loss of *MLH1* expression due to promoter methylation and microsatellite instability (MSI-H). Methylation of *Mst1* in MSI-H CRCs and their precursors, serrated polyps has previously been reported (Minoo *et al*., 2006).

Apoptosis Protease Activating Factor-1 APAF-1

Apoptosis protease activating factor-1 plays a central role in the activation of caspases involved in mitochondria-mediated apoptosis and appears to have a tumor suppressing role (Campioni *et al*., 2005). In MMR-proficient CRC, a significant difference in APAF-1 expression was observed for T stage, N stage and vascular invasion

(Zlobec *et al*., 2007a). Patients with loss of APAF-1 had a significantly worse prognosis than those retaining complete expression of the protein. Loss of APAF-1 expression was linked to the presence of metastasis in MLH1-negative CRC and correlated with worse survival time compared to tumors with complete expression of the protein. These results are in line with numerous findings in malignant melanoma that identified loss of APAF-1 as a key feature in tumor progression (Baldi *et al*., 2004). Moreover, the IHC expression of APAF-1 was found to be significantly lower in metastatic melanomas compared to tumors with no metastases (Baldi *et al*., 2004). Allelic imbalance at the APAF-1 locus was found to correlate with the progression of colorectal tumors (Umetani *et al*., 2004).

In conclusion, the immunohistochemical analysis on a large series of CRCs can be managed by including different steps that can approach more appropriately the biologic function of protein markers: (1) the randomization of the TMA dataset into two subgroups test and study group allows to test and validate the staining of protein markers; (2) the stratification by MMR status reflects more accurately the fact that CRC is a heterogeneous tumor with different prognostic significances; (3) easy, clearly defined and reproducible scoring systems avoid the use of arbitrarily set cut-off scores for positivity that often lead to contradictory results in the literature.

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15
Colorectal Cancer: Lactate Dehydrogenase (LDH) Activity as a Prognostic Marker

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LACTATE DEHYDROGENASE

Glucose is an essential source of energy for mammalian cells with the end product of glycolysis being pyruvate. For every molecule of glucose, two molecules of pyruvate are produced, while two molecules of adenosine diphosphate (ADP) are converted into adenosine triphosphate (ATP) and two molecules of NAD+ transformed into NADH (dihydronicotinamide adenine dinucleotide). This step does not depend on the presence of oxygen. Pyruvate is subsequently used for further energy production.

The fate of pyruvate is dependent on the presence of oxygen. Under aerobic conditions pyruvate enters into the mitochondria for oxidation. At these organelles, the carbon atoms of the acetyl groups are liberated, via the Krebs (citric acid) cycle, as carbon dioxide, while the hydrogen atoms are transferred to the NAD+ which, in turn, is reduced to NADH. The NADH electrons are subsequently transported into a series of molecules forming the electron-transport chain, transferring their energy to ATP, the main source of cellular energy. Finally, electrons and protons are combined with oxygen to produce water. This pathway results in the largest possible number of ATP molecules, i.e., energy, that can be obtained from pyruvate.

Under hypoxic/anaerobic conditions, however, the Krebs cycle is nonfunctional and the cells use the first step of glycolysis to gain ATP. The transformation of glucose to pyruvate for ATP production will only occur if adequate amounts of NAD+ are available. Thus, the NADH produced after glucose transformation to pyruvate is oxidized back to NAD+ in order to ensure continuation of cell glycolysis.

Lactate dehydrogenases (LDH) (Holbrook *et al*., 1975) are a group of enzymes catalyzing the reversible transformation of pyruvate to lactate. Pyruvate together with one molecule of NADH and one H+ produce lactate and NAD+ through the catalytic activity of specific LDH isoenzymes. Lactate is extruded from the cells through monocarboxylate transporters (MCTS) (Halestrap and Price, 1999), while NAD+ is used for continuing glycolysis by the cells. Although anaerobic glycolysis results in lower number of ATP production, compared to the Krebs' cycle, several tissues (such as muscle under exercise) can accelerate the glycolytic process and acquire ATP, through glycolysis, 100 times faster than that from oxidative phosphorylation. Pyruvate transformation to lactate is a reversible process facilitated by specific LDH isoenzymes. Under oxygenated conditions, lactate and NAD+ are converted

back to pyruvate that enters oxidative phosphorylation.

LDH is universally present in all mammalian cells, but specific LDH isoenzymes may prevail in different tissues according to their metabolic demands. The LDH molecule is a tetramer composed of four polypeptide chains. There are five component isoenzymes as a result of the five different combinations that are produced by two polypeptide chains encoded by separate genes (the M encoded by LDH-A gene located on chromosome 11p15.4 and the H encoded by the LDH-B gene located on chromosome 12p12.2-p12.1). The LDH-1 (H4) is composed of four H-subunits, and the LDH-5 (M4) of four M-subunits. The LDH-2, LDH-3 and LDH-4 correspond to the MH3, M2H2, and M3H isoenzymes. The prevailing type of LDH varies depending upon the tissue type. In the heart, for example, the H-gene is more active than the M-gene, the latter being strongly expressed in the skeletal muscle. As the number of the M- over H-chains increases, the LDH isoenzyme becomes more efficient in catalyzing the conversion of pyruvate to lactate (LDH-5), while an increase of H- over M-chains (LDH-1) favors lactate oxidation back to pyruvate and entrance into the Krebs' cycle.

CANCER CELLS AND THE WARBURG EFFECT

That the microenvironment of tumors is hypoxic has been known for many years and all the more it is considered to be a major cause of treatment failure, namely radiotherapy and chemotherapy (Gray *et al*., 1953; Brown, 2002). The molecular cascade triggered, as a result of cancer cell response to hypoxic stress, establishes an aggressive phenotype with increased metastatic potential and resistance to apoptotic stimuli (Rofstad, 2000; Harris, 2002). Rapid proliferation of cancer cells with high metabolic demands, defective structural and functional angiogenesis, and irregular spatial relation between the cancer cells and the stromal vasculature are the principal causes of intratumoural hypoxia (Giatromanolaki and Harris, 2001; Vaupel *et al*., 2001).

Under hypoxic stress, cancer cells are eagerly turned to anaerobic pathways for energy acquisition. Oncogenic or hypoxia mediated up-regulation of LDH-A guarantees an increased glycolytic metabolism for cancer cells and reduced dependence on the availability of oxygen (Shim *et al*., 1997; Firth *et al*., 1995). Nevertheless, for reasons rather unclear, cancer cells have an inherent tendency to turn into glycolysis even in the presence of high oxygen tension – Warburg effect (Warburg, 1931). The unveiling, at least in part, of this mystery came during the past decade when the hypoxia inducible factors 1α and 2α (HIF 1α and 2α) were identified as key transcription factors for regulating glycolysis together with the transcription of LDH-A and other enzymes involved in cellular metabolism (hexokinase, aldolase, carbonic anhydrase CA9, glucose transporters GLUTs) and angiogenesis (VEGF) (Firth *et al*., 1995; Semenza *et al*., 1996; Ebert and Bunn, 1998; Wykoff *et al*., 2001). HIFα proteins are stabilized under hypoxic conditions as their degradation, through the proteasome pathway, is suppressed (Huang *et al*., 1996). On the other hand, oncogenes active in cancer cells (i.e., the EGFR family) are able to induce over-transcription of HIFαs and trigger

the HIF cascade under aerobic conditions (Laughner *et al.*, 2001; Pore *et al.*, 2006). Elstrom and his associates showed that activation of a single oncogene, namely the Akt, is sufficient to stimulate aerobic glycolysis in tumors (Elstrom *et al.*, 2004), while the same ongogene (Akt) activates the HIF-1α (Mottet *et al.*, 2003) which, in turn, up-regulates enzymes involved in anaerobic glycolysis and glucose absorption (Semenza *et al.*, 1996). Estrogen and cyclic-AMP are also inducers of the LDH-A gene expression (Hong *et al.*, 2006; Jungmann and Kiryukhina, 2005). Figure 15.1 summarizes the pathways leading to the activation of anaerobic metabolism in cancer cells providing, at the same time, immunohistochemical images of key proteins expressed in colorectal cancer.

ACTIVITY OF LDH IN COLORECTAL CANCER TISSUES

The activity of LDH in colorectal cancer and the adjacent intestinal mucosa has been studied in freshly prepared supernatants of human tissues (Lawson *et al.*, 1989). A significantly higher activity of LDH in cancer tissues, compared to adjacent and distal uninvolved mucosa, was confirmed. Nevertheless, in approximately half of the cancer cases examined, the LDH

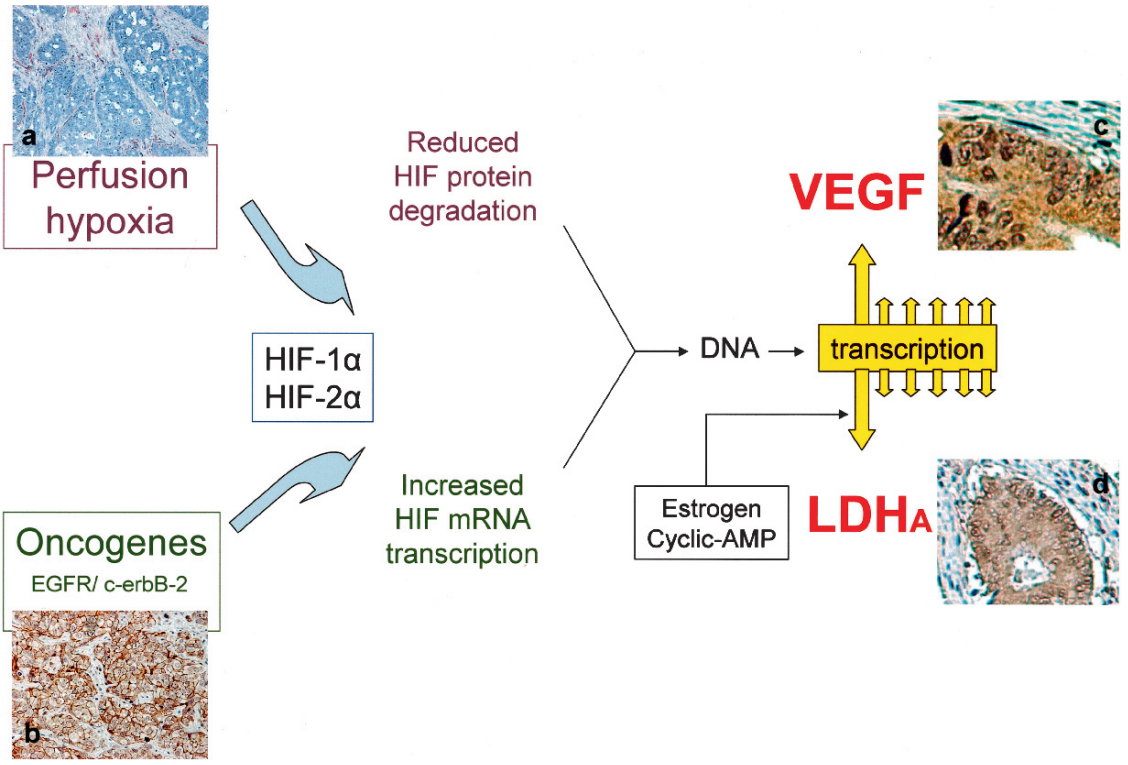

FIGURE 15.1. Pathways leading to anaerobic metabolism in cancer cells. (1a: Colorectal cancer with poor vascular density as shown by the scarse presence of CD31+ vessels. 1b: Overexpression of the EGFR in the membrane of cancer cells, 1c: Strong cytoplasmic expression of the VEGF in colon cancer cells, 1d: strong cytoplasmic expression of LDH-5 in the cytoplasm of colon cancer cells.)

levels did not exceed those of normal tissues, indicating that not all colorectal adenocarcinomas possess such enzymatic over-activity. In another study (Griffini *et al*., 1994) the activity of LDH was assessed in experimentally induced hepatic metastases, after injecting colorectal cancer cells in rats, using the neotetrazolium method. Malignant cells at the periphery of metastatic deposits displayed limited LDH activity compared to inner tumor areas. The LDH activity was particularly high at perinecrotic tumor regions.

EXPRESSION OF LDH ISOENZYMES IN COLORECTAL CANCER

The first immunohistochemical study focusing on the expression of LDH isoenzymes in colorectal cancer was that of Mate *et al*. (1993) in a series of adenomas and adenocarcinomas. The M-polypeptide was increased significantly in large adenomas, particularly when accompanied by severe dysplasia. The largest amounts, however, were found in adenocarcinomas and the authors concluded that the immunohistochemical assessment of LDH isoenzymes may be useful in detecting early malignant transformation in adenomas of the colon. Specific antibodies, recognizing the LDH isoenzymes, have recently been developed allowing a more detailed analysis of their expression in tissues. In a study by Pan *et al*. (1991) both H and M types of LDH were found in normal tissues, but M immunostaining was increased in malignant tissues.

Using the sheep polycloncal antibody 9002, raised against human LDH-5 purified from human placenta (Abcam, Cambridge, UK), and the 9001 IgG polyclonal antibody, raised against human LDH-1 purified from erythrocytes (Abcam, Cambridge, UK), we assessed immunohistochemically the expression of LDH-1 and LDH-5 in a large number of malignant and normal tissues (Koukourakis *et al*., 2003a, b). In the vast majority of cases, including colorectal adenocarcinomas, cancer cells reacted strongly with LDH-5 and, to a lesser extent, with LDH-1, while the cellular constituents of the tumor supporting stroma showed a net prevalence of LDH-1 over LDH-5 expression and, at times, complete absence of LDH-5 reactivity. This study clearly showed that colorectal cancer cells, just like all other tumor types investigated, have a high LDH-5 content indicative of a prevalent anaerobic metabolism shifting glycolysis to lactate production. The adjacent tumor associated stroma expressed LDH-5 only weakly, suggestive of an aerobic metabolic attitude of the nonmalignant tumor component. Normal colonic mucosa expressed LDH-1, but no LDH-5, consistent with an aerobic metabolism for the normal well oxygenated tissues.

The different metabolic pathways followed by cancer cells and tumor associated stroma were examined in colorectal adenocarcinomas in a subsequent study (Koukourakis *et al*., 2006b). It was shown that the colon cancer cells share enzyme/transporter activities suggestive of an anaerobic metabolism (high LDH-5, high HIFαs, suppressed pyruvate dehydrogenase PDH and overexpression of PDH kinase) with high ability for glucose absorption and lactate extrusion (high glucose transporter GLUT1 and high monocarboxylate transporter MCT1 expression).

In contrast, the tumor-associated fibroblasts expressed proteins involved in lactate absorption (high MCT1/MCT2), lactate oxidation (high LDH1, low HIFαs/LDH-5) and reduced glucose absorption (low GLUT1). Based on the above immunohistochemical metabolic profile, we suggested that the newly formed stroma express complementary metabolic pathways, buffering and recycling products of anaerobic metabolism to sustain cancer cell survival. It became apparent that tumors survive and grow because they are capable of organizing the regional fibroblasts and endothelial cells into a harmoniously collaborating metabolic domain.

SUBCELLULAR PATTERNS OF LDH-5 EXPRESSION AND SCORING OF TUMORS

LDH-5 emerged, therefore, as an enzyme of paramount importance for colorectal cancer cells sustaining metabolism, energy acquisition, survival and growth in the context of an impaired Krebs' cycle. A meticulous study of the expression patterns of LDH-5 in carcinomas would provide important information on the individual tumor metabolic attitude, probably with prognostic and therapeutic implications.

LDH-5 was expressed in the cytoplasm of cancer cells, though not in all cases and with an extent of reactivity that varied from 0% to 100% of the tissue area examined. Interestingly, nuclear shunt of the protein was also evident and, at times, quite striking – a finding also noted in previous experimental studies (Reddy *et al*., 2001). In a series of 128 colorectal adenocarcinomas stage B and C, we assessed the expression patterns of LDH-5 and scored the cases according to the grading system shown in Table 15.1 (Koukourakis *et al*., 2006a). Colorectal cancer cases with nuclear expression in > 10% of cancer cells or with strong cytoplasmic expression in > 50% of such cells were considered as being of high LDH-5 reactivity; the remaining were scored as bearing low reactivity. Overall, 77% of the cancer cases studied exhibited high LDH-5

TABLE 15.1. Grading system based on the intensity and extent of cytoplasmic and nuclear staining. Distribution of 128 colorectal cancer cases according to the patterns of LDH-5 expression.

Scoring system	No cases (%)	Score
Complete absence of reactivity	0 (0)	Negative
Weak cytoplasmic reactivity (regardless the extent)	21 (17.3)	Low
Strong cytoplasmic reactivity in less than 50% of cancer cells	8 (6.3)	Low
Nuclear expression in sporadic cells (< 10% of cells) (with strong cytoplasmic expression in < 50% of cells)	1 (0.09)	Low
Strong cytoplasmic expression in more than 50% of cancer cells	39 (30.5)	High
Nuclear expression in more than 10% of cancer cells (regardless of cytoplasmic expression pattern)	60 (46.9)	High

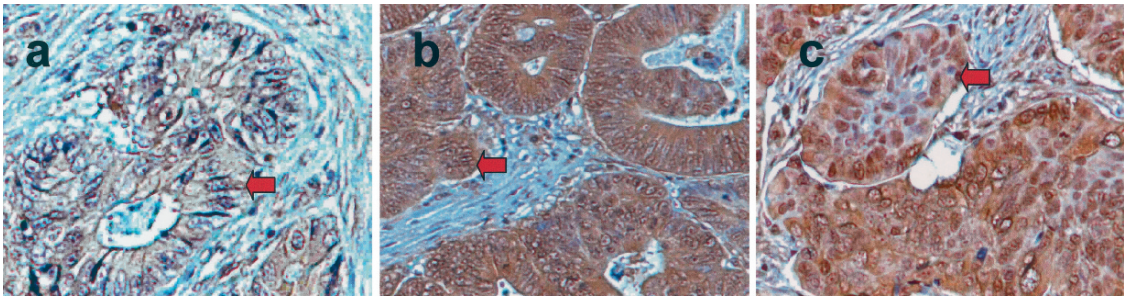

FIGURE 15.2. Immunohistochemical patterns of LDH-5 expression in colorectal cancer: (a) weak LDH-5 expresion (arrow), (b) predominantly cytoplasmic expression (arrow), (c) mixed cytoplasmic and nuclear expression (arrow)

reactivity, indicating the significance of this enzyme in the biology of colorectal cancer. Figure 15.2 shows different immunohistochemical patterns noted.

LDH AND TUMOR AGGRESSIVENESS

The aggressive tumor behaviour expected in cases with high LDH activity may be attributed to several reasons: (i) increased LDH activity leads to lactic acid production and acidification of the extracellular matrix (Vaupel *et al*., 1989; Stubbs *et al*., 2000). Low extracellular pH triggers cathepsin D and gelatinase activity, amplifying the invasive ability of cancer cells (Rozhin *et al*., 1994; Martinez-Zagulian *et al*., 1996). Moreover, macrophages are stimulated to release angiogenic factors contributing to the overall tumor angiogenicity and facilitating metastases (Murray and Wilson, 2001; Zabel *et al*., 1996; Jensen *et al*., 1986). Resistance of cancer cells to apoptosis, induced by hypoxia, is also increased by extracellular acidity, as low pH protects mitochondria from oxidative stress (Bronk and Gores, 1991; Nemoto *et al*., 2000) (ii) increased LDH production by cancer cells may also reflect intratumoural hypoxia (Firth *et al*., 1995) and, therefore, tumour resistance to radiotherapy and chemotherapy (Brown, 2002); (iii) since LDH-A is transcriptionally regulated by HIFαs, high LDH serum levels may reflect an up-regulated HIF-molecular cascade. Whether hypoxia or genetically induced HIF up-regulation results in the overexpression of proteins linked to angiogenesis/metastasis, glycolysis and resistance to apoptosis is, at present, obscure (Harris, 2002; Akakura *et al*., 2001; Semenza *et al*., 1996). There are, therefore, several reasons to explain why high LDH levels predict, directly or indirectly (HIF-dependent tumour aggressiveness), the poor postoperative outcome with resistance to cytotoxic regimens (Aebersold *et al*., 2001; Koukourakis *et al*., 2001, 2002).

LDH-5 EXPRESSION AND PROGNOSIS OF PATIENTS WITH COLORECTAL CANCER

The association of LDH-5 expression with histological and molecular variables in colorectal cancer was assessed in two series

of patients. In the first series of 75 patients treated at the Democritus University of Thrace, Greece, high LDH-5 reactivity was confirmed in 68% of cases (Koukourakis *et al*., 2005). Nuclear LDH-5 expression was associated marginally with moderate/poor differentiation and with metastases to regional lymph nodes and/or to distant organs. Using the aforementioned scoring system, high LDH-5 expression was linked with nodal and distant metastasis. Both nuclear and cytoplasmic LDH-5 expression was directly related to HIF1α accumulation, while nuclear LDH-5 was significantly related to HIF2α. In the same series of patients the authors reported a direct association between high LDH-5 expression and intense fibroblastic proliferation in the invading tumor edge (stromatogenesis), a feature linked with new blood vessel formation and deep transmural invasion in colorectal adenocarcinomas (Sivridis *et al*., 2005). It was suggested that the complicity of peritumorous fibroblasts in the overall aggressiveness/invasive and metastatic ability of the colorectal tumours is probably favored by the altered microenvironmental conditions of hypoxia and acidity, attributed, by and large, to LDH-5 activity.

In a subsequent study on 128 patients with operable colorectal cancer treated with surgery alone at the University of Oxford, UK, 77% of cases exhibited high LDH-5 expression (Koukourakis *et al*., 2006a). A significant association between LDH-5 overexpression and poor histological differentiation was confirmed. Similar to the previous series of patients, there was a significant association between LDH-5 expression and HIF1α. A striking direct association of LDH-5 with phosphorylated VEGFR2/KDR receptor expression in cancer cells and in intratumoral vessels was also confirmed, showing that LDH-5 expression is linked with activated VEGF pathway in cancer cells and tumor vasculature.

Survival analysis revealed a strong prognostic impact of LDH-5 expression (hazard ratio 15.1) in operable colorectal cancer (Koukourakis *et al*., 2006a). The projected 5-year survival was 52% vs. 96% in patients with high vs. low LDH-5 expression, respectively (Figure 15.3). This was also confirmed for stage B and C cases separately. Thus, the 5-year survival for stage B patients was 100% in the group of low LDH-5 activity vs. 67% in that of high LDH expression. The 5-year survival in stage C patients was 92% and 39% for the group of low and high LDH5 expression, respectively. In multivariate analysis LDH-5 was the most important prognostic factor, followed by vascular invasion and VEGF expression.

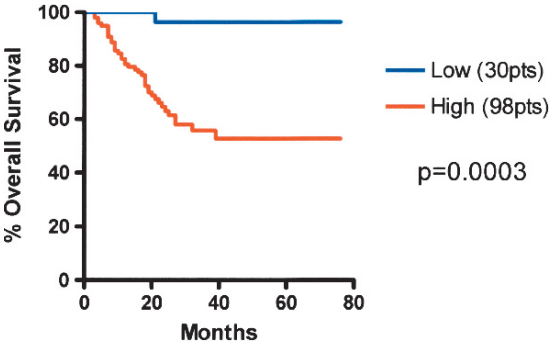

FIGURE 15.3. Kaplan-Meier overall survival in 128 patients with operable colorectal cancer treated with surgery alone (Koukourakis *et al*., 2006)

SERUM LDH LEVELS VERSUS CANCER CELL LDH-5 EXPRESSION

High serum LDH levels (> 450 IU/l) are often recorded in patients with malignancies. Serum LDH values refer to an overall level of LDH isoenzymes, as there are no conventional techniques to measure separately LDH-5 or the LDH-A subunit that reflects more accurately the anaerobic metabolic burden of tumors.

Serum levels, when fall into the normal range, represent a rather rough estimate of the intratumoural LDH-A activity as the bulk of the neoplastic disease or individual variations of LDH clearance are strong confounding factors. Yet, why LDH is released into the blood stream by tumors is unclear. Necrosis may be a mechanism, but passive or active transport cannot be excluded. Immunohistochemical assessment of LDH-5 or LDH-A, although a semi-quantitative way of assessing LDH-related anaerobic metabolism, seems to be a satisfactory approach as it allows: (i) the differential assessment of LDH-5 production by cancer and stromal cells in the tumours, (ii) the recoding of the intensity of LDH-5 activity within cancer cells, and (iii) the identification of subcellular patterns of LDH-5 localization that could be of biological relevance. Assessing LDH activity from tumor tissue extracts would be less precise since such measurements would considerably depend upon the amount of the non-malignant cellular component (stromal and reactive cells) and the extent of necrotic tissue included in the tumor sample.

In a study of patients with non-small cell lung cancer, we performed a comparative analysis of serum LDH levels before biopsy or surgery and immunohistochemical assessment of LDH-5 cancer cell expression in tissue specimens (Koukourakis *et al*., 2005). Abnormally high serum LDH levels were observed in 13/33 patients, while high LDH-5 cancer cell reactivity was noted in 20/33 of the cases studied. Linear regression analysis revealed a significant association between serum and tissue LDH, but approximately 50% of the patients with tumors showing high LDH-5 expression had serum LDH levels in the normal range. Eight days following biopsy, the mean serum LDH levels remained unchanged, while a statistically significant drop was noted in patients who underwent surgery. These findings suggest that the high LDH levels found in the sera of cancer patients is essentially of cancer cell origin. Since LDH-A is the main gene up-regulated in tumors, LDH-5 is probably the main component of serum LDH in excess.

A similar analysis was performed in a small series of 14 colorectal adenocarcinomas (Koukourakis *et al*., 2006a). Low LDH-5 expression in tumor sections was persistently accompanied by low (< 450 IU/l) LDH serum levels. Less than half of cases with high LDH-5 expression in cancer cells had high (> 450 IU/l) LDH serum levels. Statistical analysis showed a trend towards a significant association between serum and tissue LDH.

SERUM LDH-5 LEVELS AND PROGNOSIS IN PATIENTS WITH COLORECTAL CANCER

Several clinical studies have shown a significant association between serum LDH levels and prognosis in patients with malignant diseases. In addition, high serum

LDH levels define increased radio-resistance and high relapse rate after chemotherapy. In two studies from the Memorial Sloan-Kettering Cancer Center (MSKCC) (Kemeny *et al*., 1989a, b) high serum LDH levels were markers of ominous prognosis in patients with colorectal cancer. In a series of 112 patients with metastasis to liver, but not other organs, treated with infusion or systemic fluoxuridine chemotherapy, high LDH levels were associated with 8.6 months median survival compared with 18.9 months in patients with normal LDH (< 500 IU/l) levels (Kemeny *et al*., 1989a). The adverse impact of increased LDH in prognosis proved significant after multivariate analysis. Furthermore, LDH was directly linked with the extent of liver involvement by the metastatic tumor. In another study, the authors sought to investigate why patients treated with two different chemotherapy schedules from MSKCC and from the Community Clinical Oncology Program had significantly different prognosis (Kemeny *et al*., 1989b). The only single factor that could explain this finding was that the patients treated at MSKCC (poorer outcome) had more frequently abnormally increased serum LDH levels. Similarly, LDH was a significant parameter linked with poor prognosis, at least after univariate analysis, in a study from Greece where 141 patients with advanced colorectal cancer were treated with 5-fluorouracil based chemotherapy (Fountzilas *et al*., 1996).

The factors that influenced survival were examined recently in a study of 74 patients with metastatic colorectal cancer (Kuo *et al*., 2003). Multiple sites of metastasis, a high liver metastatic burden, high CEA levels, low hemoglobin and high serum LDH levels were all related significantly to poor survival. The median survival of patients with high LDH levels was 11.6 months vs. 25.5 months in patients with low LDH levels. Yuste *et al*. (2003), in a retrospective study of 91 patients with metastatic colorectal cancer, treated with 5-fluorouracil based chemotherapy, found an independent prognostic role of serum LDH levels. In an analysis of 142 patients with metastatic colorectal cancer treated with first line irinotecan or oxaliplatin based chemotherapy, high LDH serum levels were significantly related with poor outcome in multivariate analysis (Diaz *et al*., 2005). The median survival of patients with high LDH was 10.9 months vs. 18.3 in patients with low LDH. In another analysis of 45 patients, aged under 45 years, treated with adjuvant postoperative 5-fluorouracil based chemotherapy, LDH together with performance status were the only variables linked with poor prognosis in multivariate analysis (Lin *et al*., 2005). Gupta *et al*. (2005), also confirmed the unfavourable prognostic role of serum LDH in a retrospective analysis of 234 patients with colorectal cancer. In a meta-analysis, LDH was proposed as an important prognostic variable in colorectal cancer that should be systematically assessed (Watine and Friedberg, 2004).

LDH AND THE PTK/ZK TRIAL

Recently, two large randomized trials (CONFIRM 1 and 2), investigating the therapeutic value of the multi-VEGF tyrosine-kinase receptor inhibitor PTK787/ZK222584 in patients with metastatic colorectal disease treated with fluorouracil/oxaliplatin, have been concluded (Hecht *et al*., 2005). The 'confirm 1' study recruited 1,168 patients, while the 'confirm 2' study included 855 patients. In the placebo group,

serum LDH levels were strongly related to patients' survival. The 1-year/1.5-year survival in patients with performance status PS = 0 was 65/50% and 28/17% in subgroups of low and high serum LDH levels, respectively. The 1-year/1.5-year survival in patients with PS = 1 was 54/35% and 22/17% in the subgroups of low and high serum LDH levels, respectively. This is the largest performed study ever confirming the important prognostic role of serum LDH levels in metastatic colorectal cancer (unpublished data from Schering SA and Novartis reviewed by M.I.K).

Apart from this striking prognostic relevance of LDH, the "confirm" trials revealed the significance of this enzyme in predicting the efficacy of the anti-VEGF anti-angiogenic therapy in patients with colorectal cancer. The overall benefit by adding the PTK/ZK tyrosin kinase inhibitor to standard chemotherapy was marginal. Analysis of the subgroup with high levels of serum LDH showed that PTK/ZK administration led to a significant 40% reduction in the risk of disease progression. An explanation for the reason that PTK/ZK was active in patients with high LDH levels was offered from a recent investigation (Koukourakis *et al*., 2006a) where cancer cell overexpression of LDH-5 was strongly linked with activated (phosphorylated) VEGFR2/KDR receptor expression in cancer cells and in tumor associated vasculature. This association may be a result of co-regulation of the VEGF and LDH-5 genes by the key transcription factor HIF1α. Indeed, preliminary analysis of a series of neoplastic tissues from 42 patients with colorectal cancer treated in the CONFIRM trials showed that intratumoral protein expressions of LDH5, HIF-1a, pKDR and VEGFA, along with vascular density, are all significantly interrelated, supporting the concept that tumor hypoxia and angiogenesis are closely connected and that patients with elevated LDH5 protein expression have increased levels of VEGFA, activated VEGF receptors and HIF-1a (Koukourakis *et al*., 2007).

TARGETING LDH FOR THERAPY

Given the strong experimental evidence of the importance of LDH in the metabolism and survival of cancer cells and the plethora of clinicopathological data confirming the ominous prognostic role of increased serum and tissue LDH in patients treated with surgery and/or radiotherapy and chemotherapy, it is suggested that molecular interference targeting the LDH biochemical function may prove to be of therapeutic relevance.

A large number of pharmacological approaches blocking the hypoxia inducible factor HIF that regulates the expression of LDH among other genes are currently under investigation (Belozerov and Van Meir, 2006). 2-deoxy-D-glucose that inhibits the phosphorylation of glucose and disrupts the glycolysis and ATP production is expected to preferentially block the intense glycolytic activity of cancer cells overexpressing LDH (Lin *et al*., 2003; Aft *et al*., 2002). This compound (NCT00096707) is under intense clinical investigation. Specific inhibitors of the LDH-A gene have also been developed but their role in cancer therapy remains at present obscure (Yu *et al*., 2001).

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16
Detection of Tumor Cells in Lymph Nodes of Colon Cancer Patients Using Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction

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INTRODUCTION

Colorectal cancer is ranked third in worldwide incidence for women and fourth for men representing ≈ 9% of the world cancer or approximately 1 million new cases for 2002 (Parkin *et al*., 2005). Two thirds of colorectal cancers are located in the colon and one third in the rectum. At diagnosis approximately one third of all patients with colorectal cancer has lymph node positive disease, one third has lymph node-negative disease, and one third has distant metastases (Benson *et al*., 2004). The principal curative treatment for colorectal cancer is surgery. Adjuvant chemotherapy given to lymph node positive colon cancer patients has been shown to increase the survival rate (Haydon, 2003). In rectal cancer patients preoperative irradiation therapy is given to reduce local recurrences and has also been shown to improve survival (Folkesson *et al*., 2005). Still with these improved treatment modalities only approximately half the number of patients will survive for 5 years. For example, Swedish results for the time period 1995–1999 show a 5-years relative survival of ≈ 57% for both genders (Birgisson *et al*., 2005).

Tumor stage, based on histopathological examination of the resected specimen, and perioperative findings predict survival. Relative 5-year survival in Dukes' A (T1-2N0M0, Stage I) is 90–95%, Dukes' B (T3-4N0M0, Stage II) 60–80%, Dukes' C (anyTN1-2M0, Stage III) 40–60% and Dukes' D (anyTN0-2M1, Stage IV) < 5% (Staib *et al*., 2002). Besides distant metastases the most important prognostic indicator is the status of the regional lymphatic field showing presence or absence of tumor cells in regional lymph nodes. Given the importance of correctly identifying Dukes' C patients, i.e., patients with lymph node involvement who are eligible for chemotherapy, we have focused on improving the methods for detecting disseminated tumor cells in regional lymph nodes.

The histopathological method of examining hematoxylin and eosin (H&E) stained tissue section of lymph nodes for presence of tumor cells, as is performed routinely, has several drawbacks: firstly, the method lacks objectivity; this is a particularly

difficult problem when a few or a small cluster of tumor-cell like cells are observed. Secondly, because only one or a few tissue sections/lymph node is routinely analyzed only a few percent of the volume of the lymph node is actually examined. The latter problem is aggravated by the demand to examine 10–20 nodes for correct staging.

As an alternative to H&E staining we have explored the possibility of using real-time quantitative RT-PCR for detection of disseminated tumor cells in lymph nodes of colon cancer patients using mRNA for biomarkers of cancer. In two recent studies (Öberg *et al*., 2004; Ohlsson *et al*., 2006) we have analyzed a test set of lymph node mRNAs from colon cancer patients of all four Dukes' stages, from clinical controls mainly ulcerative colitis patients, primary colon tumors, normal colon epithelial cells, and a battery of cell lines including different types of immune cells. mRNA from one half of the lymph node was analyzed. Ten different biomarker mRNAs were studied including carcinoembryonic antigen (CEA), CEA cell adhesion molecule-1 (CEACAM1-S and CEACAM1-L), CEACAM6, CEACAM7-1 and CEACAM1/2, cytokeratin 20 (CK20), guanylyl cyclase C (GCC), mucin 2 (MUC2) and metalloproteinase 7 (MMP7/matrilysin). We find that real-time quantitative RT-PCR for biomarker mRNA, particularly when used with a specific probe and mRNA copy standard, is a superior method for detection of metastases and micrometastases, because it is objective, highly sensitive and quantitative, and has very wide measuring range. The major remaining task is to find the optimal biomarker or set of biomarkers, and to transform the assay into a simple plus–minus test suitable for clinical use. In this report we detail the experimental procedure, indicate a strategy for investigating whether a given marker is suitable for the detection of disseminated tumor cells in lymph nodes of colon cancer patients and give examples of results with the three most important biomarkers identified so far.

MATERIALS

Preparation of Lymph Nodes

- Sterile knives
- Liquid Nitrogen

RNA Extraction

- Stock Solution for solution D: Dissolve 250 g guanidium thiocyanate (Kodak) in 293 ml DEPC treated water, add 17.6 ml 0.75 M sodium citrate (pH 7) and 26.4 ml 10% sarcosyl at 65°C. Can be stored for 3 months at room temperature.
- Solution D: Mix 50 ml stock solution with 0.36 ml 2-mercaptoethanol. Can be stored for 1 month at room temperature.
- Sterilized glass homogenizer.
- 2 M Sodium acetate (pH 4).
- Water-saturated phenol.
- Chloroform.
- Isoamylalcohol.
- Isopropanol.
- Ethanol.
- DEPC treated water.
- Nuclease-free water (not DEPC treated) (Ambion, Cat. No 9937).
- Recombinant RNasin, 40 U/µl (Promega, Cat. No 2511).

Preparation of Copy Standard

- A DNA fragment including the qRT-PCR amplicon, cloned into a vector [pBluescript II (SK+), Stratagene] containing the T7 promoter. Note that the

amplicon has to be in the correct orientation in relation to the T7 promoter.
- Restriction enzymes, XBAI and PVUII.
- Quantum Prep, Plasmid Miniprep Kit (Bio-Rad, Cat. No 732-6100).
- NuSieve GTG agarose (BioWhittaker Molecular Applications, Cat. No 50081).
- Qiaex II gelextraction kit (Qiagen, Cat. No 20021).
- T7-MEGAshortscript, In vitro Transcription Kit (Ambion, Cat. No 1354).
- Nuclease-free water (not DEPC treated) (Ambion, Cat. No 9937).
- Recombinant RNasin (Promega, Cat. No 2511).
- NanoDrop, ND 1,000 Spectrophotometer.

Real-Time Quantitative RT-PCR of 18 S rRNA

- PCR machine; PTC-100 (MJ Research Inc).
- ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA).
- Primers and probe: Eukaryotic 18 S rRNA Endogenous Control (N4310893E) (Applied Biosystems, Foster City, CA).
- For standard curve: use for example total RNA extracted from PBMC. The concentration of the RNA should be fixed at 10,000 pg/µl (=10,000 U/µl).
- TaqMan Reverse Transcription Reagents (N808-0234; Applied Biosystems, Foster City, CA) containing MultiScribe Reverse Transcriptase (50 U/µl), RNase Inhibitor (20 U/µl), dNTP Mixture (10 mM, 2.5 mM each), Random Hexamers (50 µM), 10x RT Buffer and $MgCl_2$ solution (25 mM).
- Taqman Universal PCR master mix, No AmpErase UNG (N4324018; Applied Biosystems, Foster City, CA).
- MicroAmp Optical 96-well Reaction Plate and optical caps (N403012; Applied Biosystems, Foster City, CA).
- Nuclease-free water (not DEPC treated) (Ambion, Cat. No 9937).

Real-Time Quantitative RT-PCR of CEA mRNA, CK20 mRNA, and MUC2 mRNA

- ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA).
- Probes and primers:

  CEA probe 5'-TTC ATT TCA GGA AGA CTG ACA GTT GTT TTG CTT-3'
  Forward primer 5'-CTG ATA TAG CAG CCC TGG TGT AGT-3'
  Reverse primer 5'-TGT TGC AAA TGC TTT AAG GAA GA-3'
  Amplicon size: 82 bp
  CK20 probe 5'-CTG CGA AGT CAG ATT AAG GAT GCT CAA CT-3'
  Forward primer 5'-CGA CTA CAG TGC ATA TTA CAG ACA AA-3'
  Reverse primer 5'-GAC ACA CCG AGC ATT TTG C-3'
  Amplicon size: 82 bp
  MUC2 probe 5'-TCC CGG TTC CAC ATG A-3'
  Forward primer 5'-CCG GGC TGC TCA TTG AGA-3'
  Reverse primer 5'-TAG TGT CCA GCT CCA GCA TGA-3'
  Amplicon size: 108 bp

- The reporter dye at the 5'-end of each probe is FAM. The quencher dye at the 3'-end is TAMRA for CEA and CK20, and MGB for MUC2.
- Copystandard of CEA, CK20 and MUC2 mRNA (10^8 copies/µl).
- Unknown RNA samples.
- TaqMan EZ RT-PCR Core Reagents (N808-0236; Applied Biosystems, Foster City, CA) containing: rTth DNA Polymerase (2.5 U/µl), AmpErase UNG (1 U/µl), deoxy ATP (10 mM), deoxy CTP (10 mM), deoxy GTP

(10 mM), deoxy UTP (20 mM), 5x TaqMan EZ Buffer and Manganese Acetate solution (25 mM).
– MicroAmp Optical 96-well Reaction Plate and optical caps (N403012; Applied Biosystems, Foster City, CA).
– Nuclease-free water (not DEPC treated) (Ambion, Cat. No 9937).

METHODS

A schematic outline of the method is shown in Figure 16.1.

Preparation of Lymph Nodes

Dissect out lymph nodes from surgically removed specimens. Bisect with separate knives for each node under sterile conditions to prevent RNA cross-contamination. Immerse one half of each node in 10% buffered formalin for routine H&E staining and snap freeze the other half in liquid nitrogen and store at –70°C until RNA extraction.

RNA Extraction

The Acid Guanidine Phenol Chloroform (AGPC) method is used to extract total RNA (Chomczynski and Sacchi, 1987).

1. Weigh the tissue.
2. Homogenize tissue in 0.5 ml solution D per 25 mg tissue.
3. Transfer 0.5 ml solution to an Eppendorf tube.
4. (*) Add 50 µl 2 M NaAc (pH 4), mix by inversion.
5. Add 0.5 ml watersaturated phenol, mix by inversion.
6. Add 200 µl chloroform: isoamylalcohol (49:1).
7. Vortex vigorously for 2 min. Cool on ice for 15 min. Centrifuge at 10,000 g for 20 min at 4°C. Transfer water phase to new Eppendorf tube.
8. Repeat from (*) once.
9. Add one volume of isopropanol, precipitate at –20°C for at least 1 h.
10. Centrifuge at 10,000 g for 20 min at 4°C and discard the supernatant. Dissolve pellet in 0.6 ml of solution D.
11. Add 0.6 ml of isopropanol, precipitate at –20°C for at least 1 h.
12. Centrifuge at 10,000 g for 20 min at 4°C and discard the supernatant.
13. (**) Resuspend pellet in 75% ethanol and centrifuge at 10,000 g for 15 min at 4°C. Carefully remove the entire supernatant.
14. Repeat from (**) once.
15. Air dry the pellet for 10 min.
16. Dissolve pellet in 20 µl nuclease-free water (not DEPC treated) + 0.5 µl RNasin. Pool tubes that are derived from the same tissue sample.
17. Store at –70°C.

Preparation of Copy Standard (Fahlgren et al., 2003)

1. Prepare ≈10 µg of DNA of the cloned fragment using Bio-Rad Quantum kit.

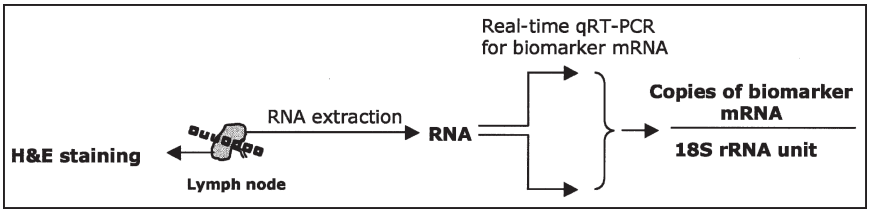

FIGURE 16.1. Schematic outline of the method

2. Cut the cloned fragment with restriction enzymes, XBAI and PVUII.
3. Separate DNA fragments by electrophoresis in 1.6% agarose gel.
4. Calculate the size of the fragment containing the qRT-PCR amplicon, and excise corresponding band.
5. Purify the fragment from the gel slice by using the QiaexII kit.
6. Add 0.1 volumes 3 M NaAc (pH 5.2) and 2.5 volumes 99.5% ethanol. Precipitate at −20°C for at least 1 h.
7. Centrifuge at 13,000 g for 20 min. Remove the supernatant.
8. Wash the pellet with 70% ethanol, carefully remove the entire supernatant and then air dry the pellet for 10 min at room temperature.
9. Dissolve the pellet in 10 µl nuclease-free water.
10. Measure the DNA concentration with a NanoDrop 1,000 Spectrophotometer (or calculate the proportion of fragment/vector and the concentration of fragment by multiplying the percentage of fragment to 10 µg [start quantity]).
11. In vitro transcribe 0.5 µg of the fragment with T7-MEGAshortscript, In Vitro Transcription Kit. Eliminate the DNA template according to protocol. Choose isopropanol for precipitation. Finally dissolve pellet in 97.5 µl nuclease-free water and 2.5 µl RNasin.
12. Measure the concentration of RNA with a NanoDrop 1,000 Spectrophotometer and calculate the number of RNA copies according to the following formula:

Number of copies/ml = (Av × m)/Mw.

Av = Avogadro's number, 6.023×10^{23}.

m = Concentration of in vitro transcript (g/ml).

Mw = Molecular weight of in vitro transcript (g/mol) =.

= (number of A × 328.2) + (number of C × 304.2) + (number of G × 344.2) + (number of U × 305.2) + 159.

The number "159" takes into account the Mw of a 5' tri-phosphate.

13. Dilute the copy standard with nuclease-free water containing RNasin to a concentration of 10^8 copies/µl.
14. Make a test run according to the qRT-PCR protocol for CEA on serial dilutions of the copy standard, for example tenfold dilutions from 10^7 to 10^1 copies/µl.
15. Evaluate the standard curve.

Real-Time Quantitative RT-PCR for 18 S rRNA

Protocol

This protocol is intended for a full 96-well plate allowing analyzes of 26 unknown RNA samples, 1 negative control and 5 standard samples all in triplicate.

Reagents	Volume	Final conc.
RT-step		
H_2O	92.6 µl	–
10x Taqman EZ Buffer	32.5 µl	1x
Magnesium chloride	71.5 µl	5.5 mM
deoxyNTPs mix	65.0 µl	0.5 mM
Random Hexamer	16.3 µl	2.5 µM
Multiscribe Rev Transcriptase	8.1 µl	1.25 U/µl
Rnase Inhibitor	6.5 µl	0.4 U/µl
RNA	1.0 µl	–
PCR-step		
H_2O	845.0 µl	–
2xTaqman universal PCR mastermix	975.0 µl	1x
20x 18 S rRNA primers and probe	97.5 µl	1x
cDNA	1.0 µl	–

1. Prepare 320 (10 × 32) µl of the RT-mix with nuclease-free water, 10x Taqman EZ buffer, magnesium chloride, deoxyNTPs mix, Random Hexamer, Multiscribe

Rev Transcriptase and Rnase Inhibitor adding the volumes shown in the protocol above. Mix by inversion and vortex gently.
2. Aliquot 9 µl of this RT-mix to 32 tubes.
3. Dilute the 18 S rRNA standard in nuclease-free water to 1,000, 200, 40, 8 and 1.6 U/µl.
4. Dilute the unknown samples to a concentration that falls within the range over which the assay gives a signal that is linearly proportional to concentration. A 1/1,000 dilution is usually appropriate when RNA is extracted as described above.
5. Add 1 µl RNA from each diluted unknown sample and 1 µl of the diluted standard samples to the tubes with the RT-mix. Mix gently.
6. Run the reverse transcription (RT-step) on a PCR machine using the following profile: 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C.
7. For the subsequent PCR-step prepare 1,920 (60 × 32) µl of a PCR-mix containing nuclease-free water, 2x Taqman universal PCR mastermix and 20x 18 S rRNA primers and probe. Mix gently and aliquot 59 µl of the PCR-mix to 32 tubes.
8. Add 1 µl of the cDNA from the RT-run to the tubes with PCR-mix and mix gently.
9. Load the 96-well plate with all 32 samples in triplicates, 19 µl/well.
10. Run the samples in the ABI Prism 7,700 Sequence Detection System (Perkin-Elmer, Wellesley, MA, USA) with the following time and temperature profile: 50°C for 2 min, 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min.
11. Determine the concentration in the samples as the mean of the triplicates defined from the results of the parallel RT-PCR analysis of serial dilutions of the total RNA standard.

Real-Time Quantitative RT-PCR for Determination of CEA mRNA, CK20 mRNA, and MUC2 mRNA

Real time qRT-PCR assays for quantitative determination of CEA, CK20, and MUC2 mRNAs were constructed in this laboratory using the Taqman EZ technology (Applied Biosystems, Foster City, CA) (Öberg *et al*., 2004; Ohlsson *et al*., 2006).

Protocol

This protocol is intended for a full 96-well plate allowing analyses of 25 unknown RNA samples, 1 negative control and 6 standard samples all in triplicate.

Reagents	Volume	Final Conc.
H_2O	864.5 µl	–
5x Taqman EZ Buffer	390.0 µl	1x
Mn Acetate	234.0 µl	3 mM
dATP	58.5 µl	0.3 mM
dCTP	58.5 µl	0.3 mM
dGTP	58.5 µl	0.3 mM
dUTP	58.5 µl	0.6 mM
Reverse primer	29.3 µl	0.3 pmol/µl
Forward primer	29.3 µl	0.3 pmol/µl
Probe	39.0 µl	0.1 pmol/µl
rTth DNA polymerase	78.0 µl	0.1 U/µl
AmpErase UNG	19.5 µl	1.0 U/µl
RNA	1.0 µl	–

1. Mix nuclease-free water, 5x Taqman EZ Buffer, Mn acetate, dATP, dCTP, dGTP, dUTP, reverse primer, forward primer, probe, rTth DNA polymerase and AmpErase adding the volumes indicated in the protocol. Mix by inversion and vortex gently.
2. Aliquot 59 µl of this mix to 32 tubes.

3. Dilute the copy standard in nuclease-free water to 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 copies/µl.
4. Add 1 µl RNA from each unknown sample and 1 µl of the diluted standard samples to the respective tubes and mix gently.
5. Aliquot 19 µl of each sample in triplicate from the tubes to the 96-well plate.
6. Run the samples in the ABI Prism 7,700 Sequence Detection System (Perkin-Elmer, Wellesley, MA, USA) with the following time and temperature profile: 49°C for 2 min, 59°C for 30 min, 94°C for 5 min followed by 45 cycles of 93°C for 20 s and 61°C for 1 min.
7. Determine the concentration in the samples as the mean of the triplicates defined from the results of the parallel RT-PCR analysis of serial dilutions of the RNA copy standard.
8. Express results as mRNA copies per unit of 18 S rRNA to be able to compare different biomarkers.

RESULTS AND DISCUSSION

In order to optimize the chances of achieving a highly sensitive and specific real-time qRT-PCR assay for a biomarker we suggest the following strategy:

- Select 2–3 forward and 2–3 reverse primers and use them in all combinations. Select the combination, which gives the best test and confirm the identity of the amplicon by sequencing. This strategy is more efficient than optimizing by changing primer and MnAc concentrations.
- Place the primers in different exons and the probe over the exon-boundary. In this way a DNA signal is excluded. If possible, see that the most discriminating sequence is located at the 3'-end of the primers. An amplicon size of 70–120 nucleotides is preferred.
- Use the TaqMan EZ RT-PCR Kit rather than first preparing a cDNA library and then running the PCR. By using the targeting primers in the RT-step even low levels of the relevant mRNA will be converted into cDNA.
- Prepare a copy standard for each biomarker mRNA. This will enable comparison of different biomarkers with respect to relative levels of expression. This is of importance as an abundantly expressed message is generally preferred as a biomarker mRNA.

To evaluate whether a new promising biomarker indeed is useful for the detection of disseminated tumor cells in lymph nodes of colon cancer patients we start with applying the new marker assay to a set of 20 primary colon cancer tumors representing all four tumor stages. The result with ten biomarker mRNAs is shown in Figure 16.2, and the median value is given in Table 16.1. Two types of relevant information are obtained: the relative expression level of each biomarker mRNA and the variation between individual primary tumors. From this data set it can be seen that CEA mRNA is expressed at ≈10 times higher levels than any of the other markers followed by a group including CEACAM1-S, CEACAM6, CEACAM7-2, CK20 and GCC mRNAs at ≈10 times lower concentrations. CEACAM1-L, MUC2 and MMP7 mRNAs are expressed at ≈100 times lower concentration than CEA mRNA and CEACAM7-1 at very low levels. MUC2 mRNA and CEACAM7-2 mRNA showed the largest variation between different primary

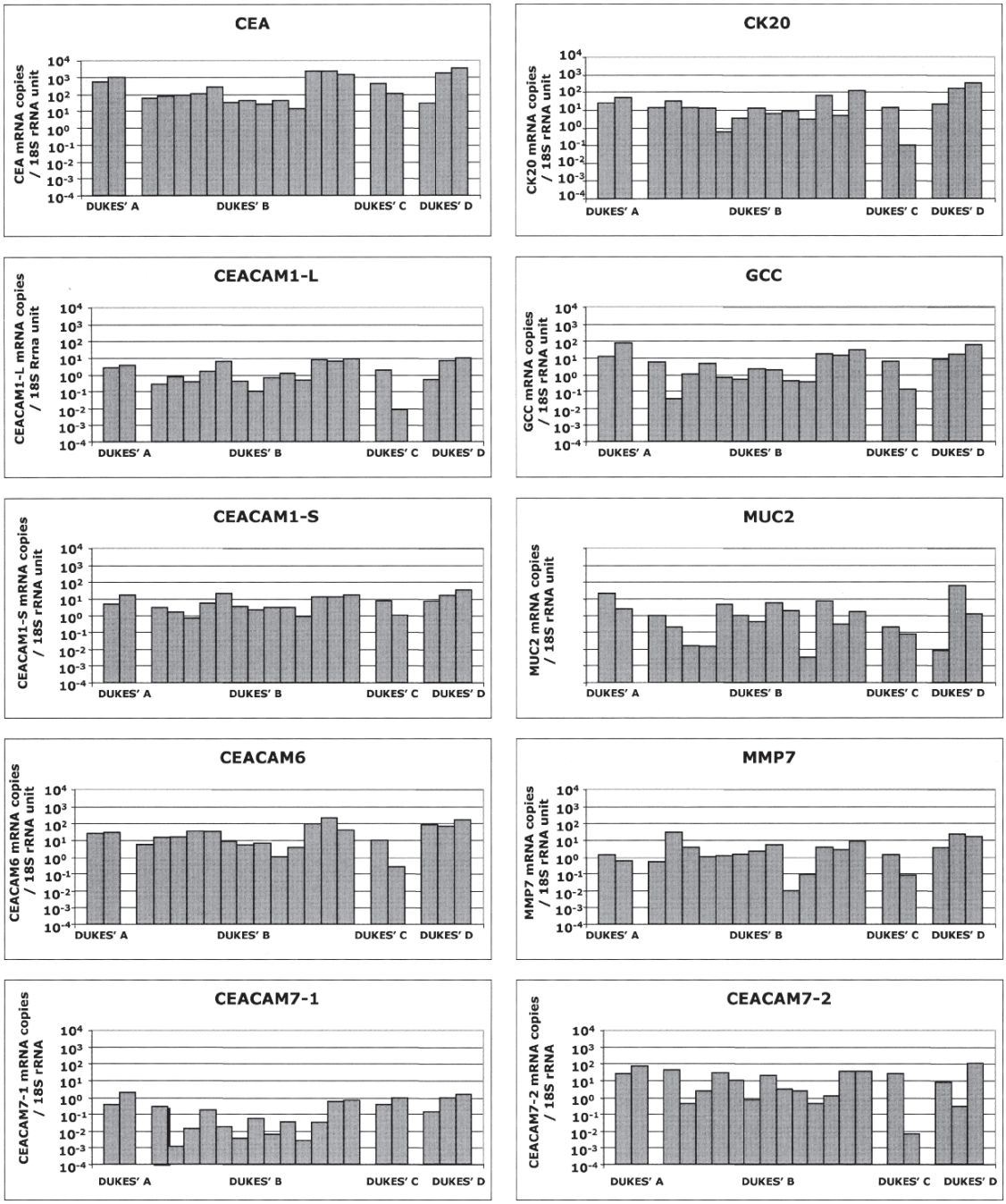

FIGURE 16.2. Expression of biomarker mRNA in primary tumors from colon cancer patients

tumors. The other biomarker mRNAs were all similar in that they showed less variation between samples.

Next we studied mRNA expression of the ten biomarkers in isolated colon epithelial cells (iECs) (Ohlsson *et al.*,

TABLE 16.1. Expression levels of biomarker mRNA in primary colon cancer (CC) tumors, normal colon epithelial cells (iEC), CC cell lines, peripheral blood mononuclear cells (PBMC) and immune cell lines.

			CEACAM1-S		CEACAM6		CEACAM7-2		MUC2		GCC
Cells	Origin	CEA		CEACAM1-L		CEACAM7-1		CK20		MMP7	
CC tumors[a]	Colon iEC	107[b]	5.1	1.4	20	0.05	9.0	14	0.66	1.7	4.9
Normal colon iEC[c]	Colon iEC	261/393	19/28	16/22	28/38	0.9/1.9	302/483	295/162	32/33	0.09/0.09	5.5/5.9
HT29	Colon iEC	32	22	17	43	0.003	0.3	85	0.01	53	0.004
LS174T	Colon iEC	328	1.2	1.4	81	0.5	38	0.02	4.3	3.4	3.8
T84	Colon iEC	33	0.9	0	1.0	0.003	0.07	33	0.5	2.3	12
HCT8	Colon iEC	32	0.003	0	0.5	0	0.1	0.05	0.02	0.2	0.05
PBMC	Blood	0	0	0.3	0.05	0.01	0	0	0	0.001	0.002
Act. PBMC	Blood	0	0.001	0.8	0	0	0	0	0	2.0	0.003
Jurkat	T cell	0	0.004	0.1	0	0	0	0	0	0.004	0.002
Molt-4	T cell	0	0.002	0.005	0	0	0	0	0	0	0.002
CNB6 + KR4	B cell	0	0.008	1.1	0	0.05	0	0	0	0.6	0.04
U266	Plasma cell	0	0.001	0.02	0	0	0	0	0	0	0.02
U937	Monocyte	0.005	0.002	0.03	0.3	0	0	0.003	0	0.9	0
HL60	Granulocyte	0	0	ND	1.8	0	0.001	0	ND	0	ND
K562	Pre-erythrocyte	0	0.006	0.08	0	0	0	ND	0	0	0

[a] Twenty primary colon cancer tumors were analysed (2 Dukes' stage A, 13 Dukes' stage B, 2 Dukes' stage C, and 3 Dukes' stage D).

[b] Median values of mRNA copies/18 S rRNA unit.

[c] Normal colon iEC of crypt-iEC fraction/luminal-iEC fraction. For further detail see Ohlsson *et al*., 2006. ND = not done.

2006) and in four different colon adenocarcinoma cell lines (Table 16.1). Only one of the biomarker mRNAs, namely MMP7 mRNA is actually upregulated in tumors compared to normal colon epithelium. mRNA for three biomarkers, CEA, CEACAM6, and GCC were expressed at approximately the same levels in normal colon epithelium and colon carcinomas. The remaining six biomarker mRNAs, including CEACAM1 mRNA (both splice forms), MUC2 mRNA, and CK20 mRNA were actually down-regulated in the tumor. Clearly it is a disadvantage that the biomarker mRNA is down-regulated in the tumor cells. Analyses of the four colon carcinoma cell lines revealed large heterogeneity of expression for all markers except for CEA mRNA indicating that essentially marker-negative colon cancer lymph nodes containing tumor cells may occur with the other nine markers.

Because tumor cell mRNA is assayed in lymph nodes in which immune cells dominate, we investigated whether immune cells would express these marker mRNAs. The results are shown in Table 16.1. Four biomarker mRNAs were not expressed, or hardly at all expressed, in any type of immune cells namely CEA, CEACAM7-2, CK20, and MUC2. Three biomarker mRNAs, CEACAM1-L, CEACAM6, and MMP7 would appear to be disqualified as biomarker for colon cancer tumors in lymph nodes because they were expressed at high levels in one or more types of immune cells. The other three markers showed low expression in some types of immune cells. Whether these low levels are of importance in practice is difficult to determine.

All ten marker assays were then used to investigate a test set of mRNA samples extracted from lymph nodes of colon cancer patients of all four Dukes' stages and from colonic lymph nodes of patients with benign disease. The latter mRNA samples (n = 83 derived from 19 patients with benign bowel disease) were used to establish a clinically relevant cut-off level that was set to two times the highest values of any of the control lymph nodes.

Figure 16.3 shows the result for the three best biomarker mRNAs. In the graph only the result of the node expressing the highest mRNA value for each patient is shown. Filled circles indicate that the particular node was positive with H&E staining and the open circles was H&E negative. This graph summarizes the result of analysis of 431 lymph nodes from 136 colon cancer patients (as an average, 3 nodes/patient). The average observation time after operation was 37 months. During this time two Dukes' A patients and five Dukes' B patients have died from colon cancer (indicated by arrows in Figure 16.3). The results can be summarized as follows: (1) The CEA mRNA assay detected all H&E positive nodes except one. The CK20 assay missed three and the MUC2 assay missed seven H&E positive nodes. (2) With all three biomarkers, but in particular with CEA, a number of Dukes' B patients and some Dukes' A patients had lymph nodes which expressed high levels of mRNA clearly above the cut-off level. All these tumor cell positive nodes were missed by H&E staining. (3) Of the five Dukes' B patients that have died from colon cancer during the observation period, four showed CEA values clearly above the cut-off level, three showed elevated CK20 values, and two showed elevated MUC2 values. None of the two Dukes' A patients were detected with any of the three markers.

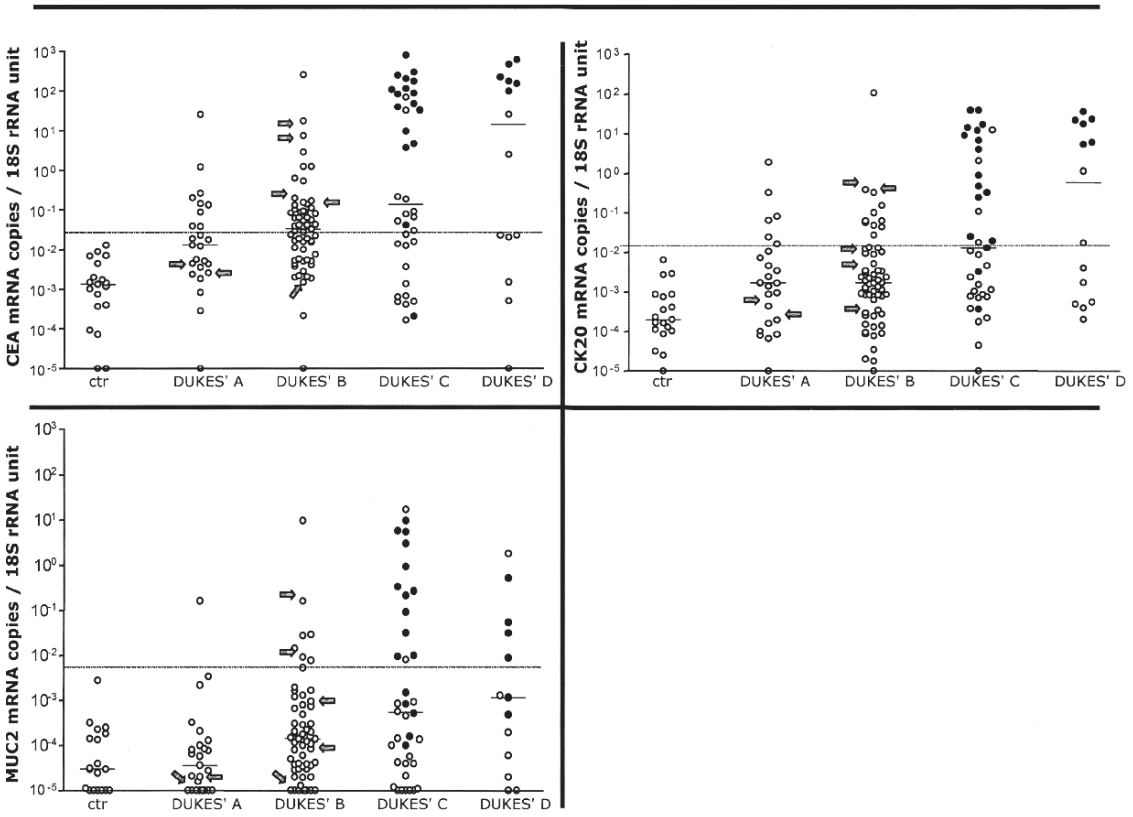

FIGURE 16.3. Expression of CEA, CK20, and MUC2 mRNAs in lymph nodes of patients with colon cancer

The other markers performed significantly worse and the CEACAM1-S/L and CEACAM7-1/2 and MMP7 biomarkers showed almost no discriminating power between colon cancer nodes and control nodes confirming the predictions from the results detailed above (for further details see Ohlsson *et al*., 2006).

In conclusion we demonstrate that the real-time quantitative RT-PCR technique described here is a sensitive and specific method for detection of tumor cells in H&E-negative lymph nodes of colon cancer patients. CEA mRNA was the most sensitive and specific among ten tested biomarker mRNAs for detection of tumor cells in lymph nodes. Follow-up data demonstrated that tumor recurrences and cancer specific deaths predominantly occurred in patients with elevated CEA mRNA levels in H&E-negative lymph nodes of the resected mesenterium. CEA mRNA may serve as a complementary selection marker for adjuvant chemotherapy treatment besides routine histopathology for disseminated tumor cells.

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A. Treatment

17
Colon Cancer: Laparoscopic Surgery

David W. Larson and Heidi Nelson

INTRODUCTION

The operative choices available for colectomy for cancer expanded in May of 2004 with the publication of Clinical Outcomes of Surgical Therapy (COST) (Nelson and COST Study Group, 2004). Laparoscopic or minimally invasive surgery (MIS) for the treatment of malignant disease had the evidence needed to ethically offer it to patients with cancer. Although laparoscopic surgery for colonic disease languished with relatively slow adoption in the 1990s and early 21st century, it has more recently gained momentum. This interest in MIS has been due in large part to the growing and expanding array of surgical indications given trials such as COST, Conventional vs. Laparoscopic-Assisted Surgery in Patients with Colorectal Cancer (CLASICC), and Colon Cancer Laparoscopic or Open resection (COLOR) (Nelson and COST Study Group., 2004; Guillou *et al*., 2005; Veldkamp *et al*., 2005; Jayne *et al*., 2007; Fleshman *et al*., 2007) as well as the improvement in instrumentation and surgical education. Both the growth in clinical indications and academic publication and education has altered the tenor of surgical debate, to one committed to minimally invasive approaches. However, there remain many important issues which the surgeon must weigh in order to properly implement these modern techniques in the setting of colon cancer.

The use of laparoscopic surgery for colonic cancer requires much thought and consideration. Technically, the surgery is much more demanding than its open counterpart. Although the actual details of the operation have changed little from the open approach, the integration of imaging, instrumentation, and surgical expertise have added a dimension of complexity to the operation that many surgeons find unfamiliar. As one overcomes these significant obstacles, the results for the patient are obvious with multiple publications demonstrating improved postoperative morbidity and improved recovery (Nelson and COST Study Group., 2004; Guillou *et al*., 2005; Veldkamp *et al*., 2005; Noel *et al*., 2007; Lacy *et al*., 2002). Here we hope to articulate the presentation and staging, risks and complications, oncologic outcomes, and techniques regarding the use of a laparoscopic approach in the treatment of colon cancer.

PRESENTATION

Adenocarcinoma of the colon often presents with little warning. When signs or symptoms exist, they often take the form of occult bleeding and or anemia, partial obstruction, and on occasion a palpable

mass. Because of these limited signs and symptoms, many patients learn of their cancer through routine screening methods or through work up required for their limited symptoms. Historically, surgeons were concerned with MIS due to its lack of tactile sense, and thus a lack of proper intraoperative staging. This controversy created a heightened sense of importance regarding the preoperative work-up and staging to aid laparoscopic surgeons in their ability to identify metastatic disease.

This issue of unsuspected metastatic disease (M1) was resolved by recent level one evidence which found the risk of unsuspected M1 disease at the time of operation to be 1–4% (Nelson and COST Study Group., 2004; Guillou *et al*., 2005; Veldkamp *et al*., 2005). With these data in hand, we have standardized our preoperative staging for all patients undergoing a laparoscopic colectomy for cancer. Prior to surgery we routinely mark or tattoo the surrounding colonic wall of any tumor in which the location is not assured. Standard work-up also includes complete colonoscopy to confirm pathologic diagnosis and clear the rest of the colon, which has a 3–5% rate of synchronous cancer. Additional workup should include a chest X-ray, complete blood count, liver-function tests, and carcinoembryonic antigen measurement. To aid our ability to assess for M1 disease, a computer tomography (CT) of the abdomen and pelvis has become our diagnostic test of choice to fully evaluate patients for intraabdominal metastases, locally advanced disease, and specifically liver metastases. Considerations must also be given to the possibility of familial syndromes like Hereditary Non-polyposis Colon Cancer (HNPCC) and the need for genetic and micro satellite instability tests in selected patients with strong family history, early onset, and right sided lesions.

Currently, only direct invasion of surrounding structures (T4 tumor) that are not amenable to a laparoscopic approach are a contraindication. When one encounters such a locally aggressive tumor, involving adjacent organs such as duodenum, small bowel, or retroperitoneal structures (i.e., ureter or gonadal vessels) every attempt must be made to complete an enbloc resection. At no time should a surgeon attempt to surgically separate intraabdominal structures from the tumor, as this would violate oncologic surgical principles and potentially adversely affect patient outcome. A curative resection (R0) is the goal of every operation. Other relative contraindications include the presence of a bulky tumor which exceeds 8–10 cm in size which makes the use of laparoscopic surgery less appealing given the large incision which will be required to remove the tumor negating any benefits of the laparoscopic approach.

In the rare chance that unresectable disease is found intra-operatively despite preoperative work-up, our practice has been to resect the primary tumor to prevent the future possibility of hemorrhage or obstructive complications. If the surgeon feels the primary tumor cannot be removed safely, a bypass procedure or stoma should be performed to prevent future obstruction.

COMPLICATIONS

The best data to date would suggest that morbidity associated with a laparoscopic colonic resection for cancer should be 20% with 2–4% of these occurring intraoperatively

(Nelson and COST Study Group, 2004; Veldkamp *et al*., 2005). Most complications from this operation can be classified into three areas; those related to MIS specifically; those common to all operations of the colon; and those related to the malignant process itself.

Complications related to MIS specifically result from improper use of laparoscopic instrumentation or energy delivery devises. Advances in vessel sealing and laparoscopic dissection devices have helped surgeons to rapidly improve their abilities to mobilize and ligate large vessels. These newer devises have significantly improved results and decreased the level of complexity associated with surgical resection. However, complications related to these high energy devices still occur and are typically related to bleeding and thermal injury to associated bowel. Although surgeons tool box contains important tools, these instruments must be handled with the attention to detail and meticulousness that ensures a safe and complication free operation. Vessel ligation in the setting of cancer is critical to allow for proper mesentery dissection and lymph node harvest. In our opinion it is important for the operating surgeon to be able to technically address the issue of vessel ligation. Standard surgical practice of traction and counter traction, separation of important structures, and a keen understanding of spatial distance are the key factors which prevent unwanted thermal or energy related complications.

Beyond the pitfalls of energy devises and vessel ligation, laparoscopic patients are at risk for injuries common to all operations of the colon and rectum including: bleeding (1–4%), bowel injury (1–2%), ureteral injury (1%), wound infection and dehiscence (2–5%), anastomotic failure (2–3%), deep vein thrombosis (1–2%), and death (0.5–5%) (Nelson and COST Study Group., 2004; Guillou *et al*., 2005; Veldkamp *et al*., 2005). Long term risks such as injury to the pelvic autonomic nerves are most important to consider during rectal mobilization in the setting of sigmoid cancer. During this time it is critical that the surgeon protect the hypogastric nerves to reduce the incidence of retrograde ejaculation and the nerves which potentially lead to impotence along the anterolateral dissection of the rectum. Concern has been raised about possible increase rates of pelvic nerve injury leading to bladder and sexual dysfunction with the use of laparoscopic surgery. A single large randomized controlled trail has published short term outcomes regarding some of these issues in rectal cancer (Guillou *et al*., 2005). Jayne *et al*. (2005) who utilized the patients in the CLASICC trial showed a significant decrease in sexual function and erectile function in men after laparoscopic rectal cancer surgery and suggested an increased concern in this regard. Long term data from this trial and future studies will no doubt weigh heavily on this unresolved debate.

Initial complications related to oncologic concern arose mainly from the risk of trocar site implants and the inability to adequately assess the abdomen for metastatic disease. Trocar site recurrence has been reported as high as 21% (Berends *et al*., 1994), although in the setting of well trained surgeons this percentage should approach only 1% (Nelson and COST Study Group, 2004). This rate of recurrence is similar to that seen in open surgery. The second issue is one which has already been discussed and involves the

risk of unsuspected M1 disease. Although failure to detect metastatic disease is a risk, with proper preoperative work-up, this risk remains small and can be attested to by the results of the COST, CLASICC, and COLOR trials (Nelson and COST Study Group, 2004; Guillou *et al*., 2005; Veldkamp *et al*., 2005).

OUTCOMES IN CANCER

The concern initially regarding laparoscopic cancer surgery was port site recurrence. Reports of recurrence were strikingly high in the early days of laparoscopic colectomy with incidence up to 21% (Berends *et al*., 1994). Subsequent larger series including COST reported a more realistic incidence of 0–1.2% (Nelson and COST Study Group, 2004; Fleshman *et al*., 2007). This is comparable to that seen in open surgery. The issues of recurrence and survival in colon cancer have been most convincingly addressed by the COST trial (Nelson and COST Study Group, 2004; Fleshman *et al*., 2007). This study included 48 institutions and 66 surgeons who all performed more than 20 laparoscopically assisted colorectal operations prior to the study. After a median follow-up of 4.4 years, recurrence and survival rates (overall and disease-free) were found to be equivalent for both groups (Figures 17.1–17.3) (Fleshman *et al*., 2007). This study provided major evidence to confirm the safety of laparoscopic-assisted surgery for colon cancer. Other randomized controlled trials including COLOR and CLASICC have yet to report their long term 5-year survival data.

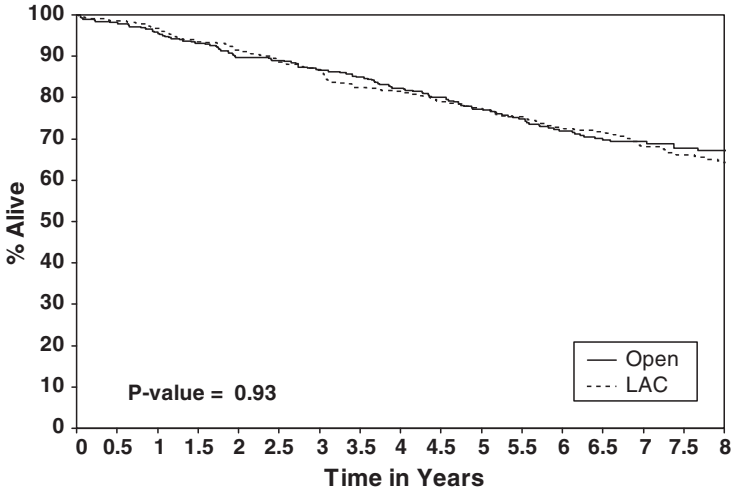

FIGURE 17.1. Overall survival

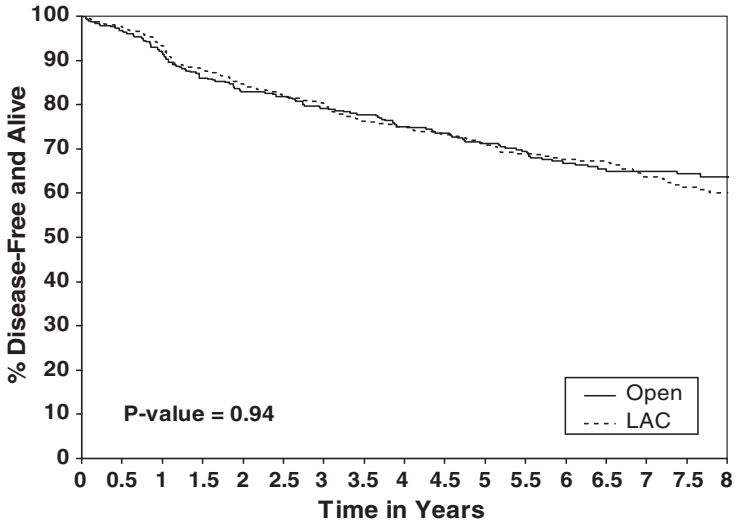

FIGURE 17.2. Overall disease free survival

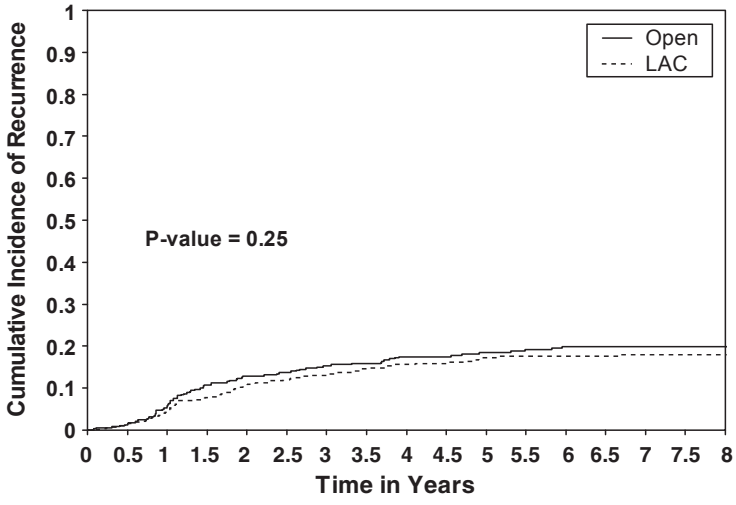

FIGURE 17.3. Overall local incidence of recurrence

TREATMENT TECHNIQUE OF OPERATION

The dissection and resection of a large intraabdominal organ involving all quadrants of the abdomen and pelvis require the operator to overcome many technical challenges. The fact that the colon and rectum have a large and extensive blood supply requires the development of special instruments to achieve intracorporeal division of blood vessels. The ability to move and mobilize the shear mass of this large organ in the abdomen requires a significant level of expertise. Therefore, if one is unable to fulfill the requirements for a proper oncologic resection one must admit that conversion is in the patient's best interest. We now have a gold standard or base line to measure our individual results against, realizing that studies like COST have defined these benchmarks such as: the length of bowel margin, points of vascular ligation, lymph node harvest,

and resection length. The data from these trials have demonstrated that laparoscopic resection completed by well trained surgeons can be achieved without compromising these factors. It is important that each of us who performs these operations critically examine and review our own cases assuring ourselves and more importantly our patients that proper benchmarks are being followed.

The type of laparoscopic approach used to achieve these objectives has added a wrinkle into the fabric of this debate. The traditional methods of totally laparoscopic or laparoscopic assisted surgery now also include hand-assisted surgery (HALS). HALS uses an abdominal wall port, large enough to admit the operator's hand, which provides an airtight seal allowing maintenance of a pneumoperitoneum. The traditional difficulty with retraction, lack of tactile discrimination and learning curve are significantly improved with this technique. To date, the results of studies performed reveal that the postoperative patient related outcomes of laparoscopic surgery are not compromised (Davies and Larson, 2004) based on this new technique. No studies however, have been published of any significance which shed light on the oncologic outcome of HALS versus more traditional laparoscopic techniques.

Exploration

Exploration is a critical step in the surgical management of both benign and malignant disease. It remains most critical for malignant disease as rates of unsuspected M1 disease range from 1–4% (Nelson and COST Study Group, 2004). Once the abdomen is entered, a thorough exploration of the abdomen is the first order of business. It is required that liver be palpated or visualized in the case of laparoscopic surgery, the gallbladder must be assessed for stones, and the surrounding organs of the upper and mid abdomen are examined. The small bowel is run from the ligament of Treitz to the ileocecal valve. In women, the uterus and ovaries must be inspected for any pathology as metastatic disease may occur in up to 3% of patients. One must remember that intra-operative ultrasound can also be used to enhance the hepatic evaluation for metastasis. During exploration we determine whether adhesions, altered anatomy, or tumor characteristics will require conversion to open surgery. If so, conversion is performed promptly.

Right Colectomy

Regardless of the operation, positioning the patient begins with tucking, padding, and protecting both arms at the side. Given the multiple changes of position, we commonly use ankle and chest straps with the patient positioned supine to secure the patient properly.

The line of resection for a right colon cancer depends somewhat on the location of the tumor. For those tumors located in the cecum, a margin of terminal ileum is generally taken. This line of resection should extend to the right side of the transverse colon at the level of the right branch of the middle colic vessels (Figure 17.4). Care must be taken to preserve the main branch of the middle colic vessels. The right colic and ileocolic vessels are taken at their origins to ensure proper lymph node harvest. Omental attachments to the right colon are generally removed with the specimen.

The laparoscopic operation commences with the placement of the first port

(10/12 mm) using a cut down method in the supraumbilical position. Through this a 30° optic device is utilized. The abdomen is insufflated with carbon dioxide to a pressure between 12–14 mmHg. The second and third trocars are placed under direct vision using two 5 mm trocars. These trocars are placed in the left lateral and suprapubic position (Figure 17.5).

The patient is now placed in the Trendelenburg position with the right side tilted up while the surgeon and the assistant stand on the left (Figure 17.6). The first step of dissection is ureter identification, which can usually be done at the level of the pelvic brim. In obese patients one must first score the peritoneum to identify the ureter. Traction and counter traction are the key factors in proper anatomic dissection of the large intestine. Additional safety can be gained by only grasping the peritoneum surrounding the bowel, thus avoiding direct contact with the bowel. By placing the proper traction on the right colon one can mobilize the colon separating it from the retroperitoneal structures (gonadal vessels and ureter). The first step in this is performed by incising the peritoneal attachments laterally allowing one to rotate the cecum superiorly and medially (Figure 17.6). Once this mobilization is completed the medial attachments between the retroperitoneum and the right colon and terminal ileum are incised up toward the junction of the third and fourth portions of the duodenum (Figure 17.7).

Mobilization of the hepatic flexure occurs by dissecting from left to right in the proper plane. The patient is placed in reverse Trendelenburg with the left

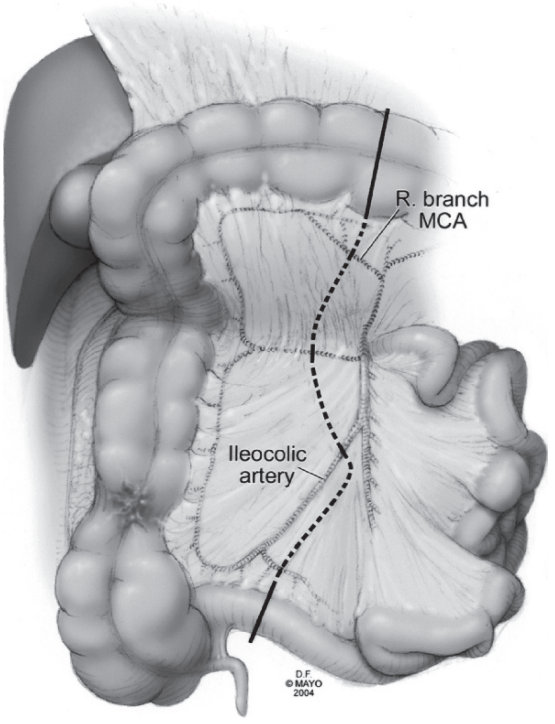

FIGURE 17.4. Line of resection for a right hemicolectomy for cancer

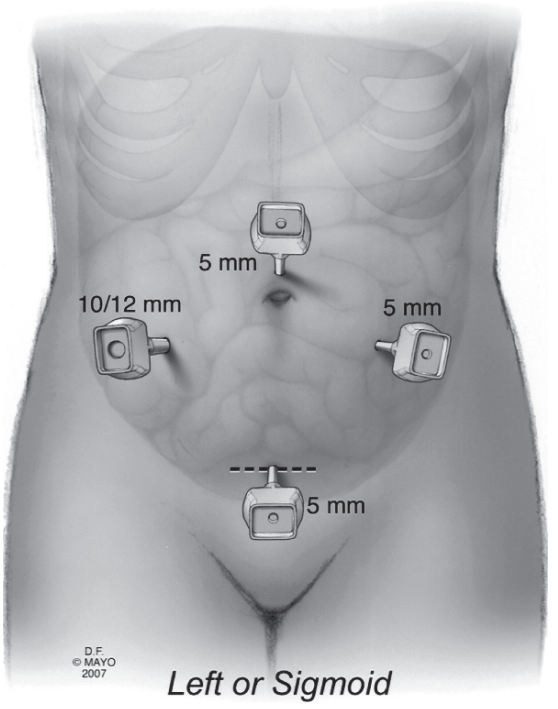

FIGURE 17.5. Port position and exteriorization incision for right sided operations

side down. Mobilization of the colon at the hepatic flexure should start along the free peritoneal edge of the transverse colon. The gastro-colic ligament is grasped near but not on the bowel, and elevated toward the anterior abdominal wall and the feet. This thin ligament can often be separated from the deeper tissues of the colonic mesentery. Retracting the mid transverse colon inferiorly, one can expose the hepatic flexure allowing the surgeon to separate the colon from other important structures like the duodenum and kidney (Figure 17.8). Once in the correct plane the thin attachment between the mesocolon and the gastrocolic ligament can be divided quickly completing the flexure mobilization.

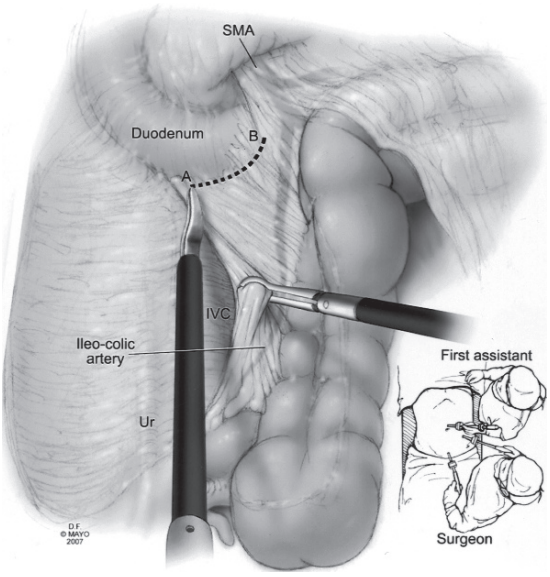

FIGURE 17.7. Medial and inferior mobilization of the right colon

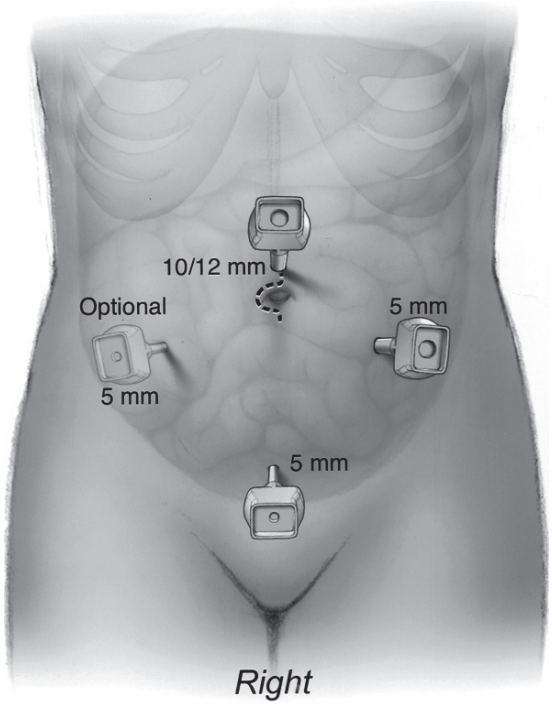

FIGURE 17.6. Surgeon and first assistant positions, and lateral to medial mobilization of the right colon

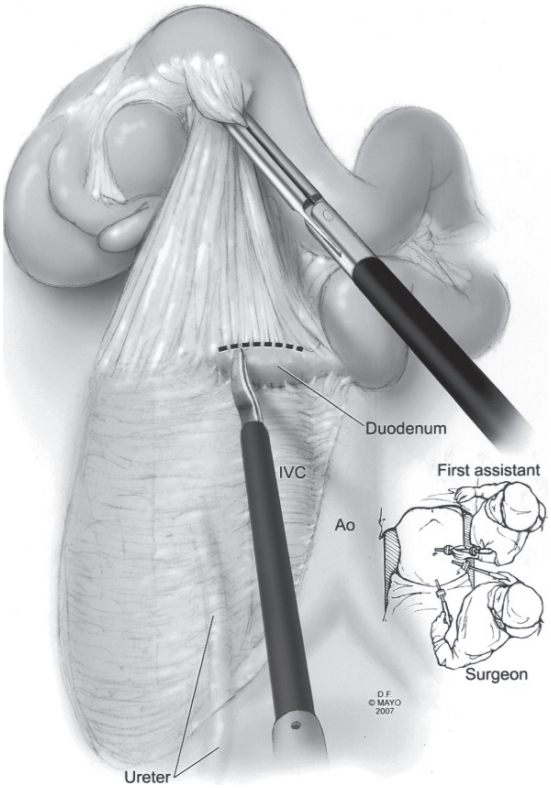

FIGURE 17.8. Hepatic flexure mobilization

Once fully mobilized, vascular control is achieved by applying moderate tension on the junction of the ileum and cecum, with a Babcock through the supra pubic port. This allows one to readily display the ileo-colic and right colic vessels facilitating intracorporeal ligation. It is critical that when completing this dissection, one transects these vessels at the root of their take off from the SMA. In order to achieve this the peritoneum overlying the ileocolic vessels is incised and then the vascular pedicle is secured by ligasure™ or linear vascular stapler, through the left lower quadrant port (Figure 17.9). Of course, when an energy sealing device is used, one can avoid a larger trocar which would be required if a stapler was to be deployed. The marginal branches to the ileum can be divided next, thus preparing the proximal line of resection. The final step, if needed, includes taking the right branch of the middle colic artery completing the intracorporeal dissection. Alternative approaches have also been described previously by our institution (Young-Fadok and Nelson, 2000).

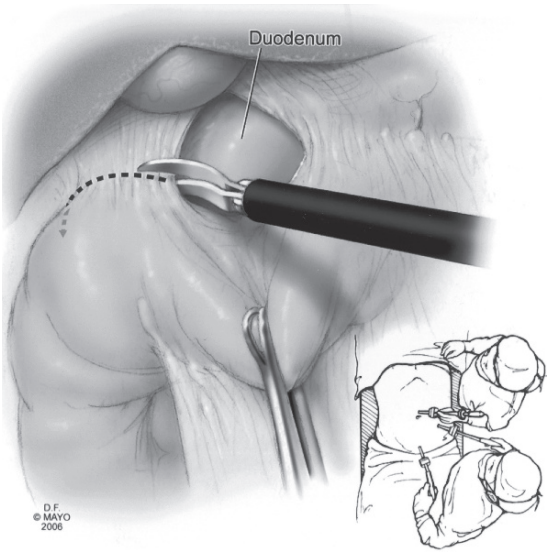

FIGURE 17.9. Ileocolic vessel ligation

In order to exteriorize the right colon, the incision at the supraumbilical port is enlarged around the umbilicus for about 3–6 cm depending on the size of the patient and the specimen. All wounds are protected with a wound protecting device, and the pneumoperitoneum is released through the trocars in a controlled manner. Once exteriorized it is important to maintain mesenteric orientation at all times for a proper anastomosis. The resection and anastomosis are performed in a standard manner, respecting appropriate proximal and distal margins. The mesenteric defect may be closed or left open. The bowel is returned to the peritoneal cavity resuming its normal anatomic position. It is best to run the small bowel at this point to assure that the colon is positioned in the retroperitoneum and the small bowel is not trapped behind the new anastomosis.

The Anastomosis

The anastomosis itself can be created in multiple ways. We typically use one of two basic techniques which have served us well. These two categories of anastomosis can be employed in nearly any colon resection and include either a hand sewn or a stapled anastomosis. The hand sewn anastomosis begins by placing crushing bowel clamps across the colon a few centimeters proximal to the area to be divided as well as few centimeters distal to the line of transaction in the ileum (Devine and Pemberton, 1995). Non-crushing clamps are then placed straight across the colon and ileum to be preserved to prevent spillage of intestinal contents. At this point the colon and ileum are divided and the specimen is sent to pathology. If the diameter

of the transected bowel is small, as it often is with the terminal ileum, it can be enlarged by dividing it longitudinally along its antimesenteric boarder (the so-called Cheatle cut). A two-layered anastomosis can be performed in an end to end or end to side fashion as illustrated in Figures 17.10–17.13. First, the two ends of the bowel are approximated, making sure there are no twists. 3–0 stay sutures are utilized in the corners of the bowel to aid with approximation. A posterior row of Lembert sutures is placed first. These sutures should be placed deep enough to incorporate most of the muscle layer. If the suture can be seen through the serosa, then the stitch has been placed too superficially and a deep needle passage is required. The sutures are tied to approximate tissues, not to strangulate them. Next an inner layer of running 3–0 suture is used to approximate the mucosal and submucosal layers. The corner of the bowel is secured first and the running suture is then advanced along the

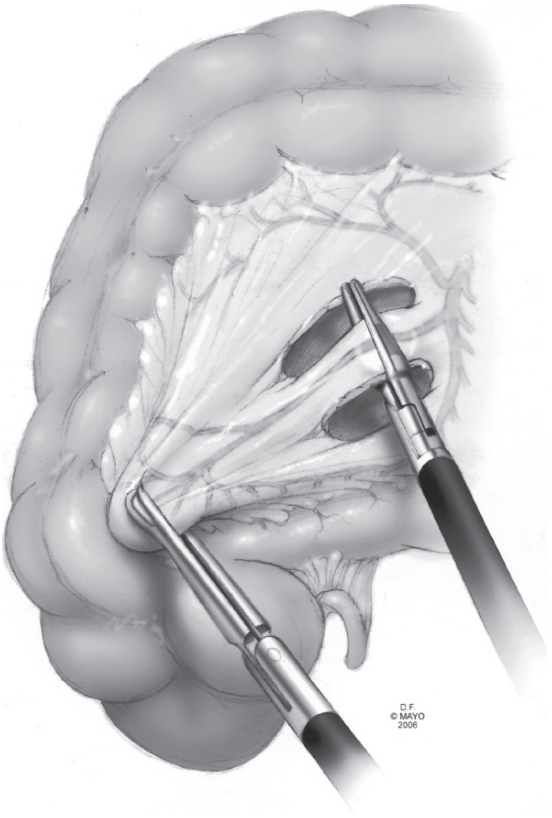

FIGURE 17.10. Posterior interrupted row of a hand sewn anastomosis

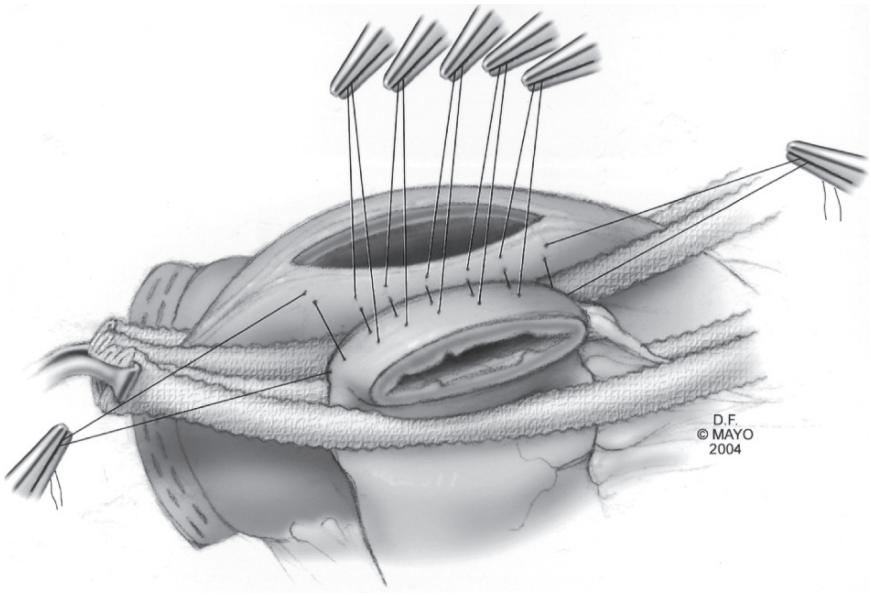

FIGURE 17.11. Posterior row of the inner running layer of a hand sewn anastomosis

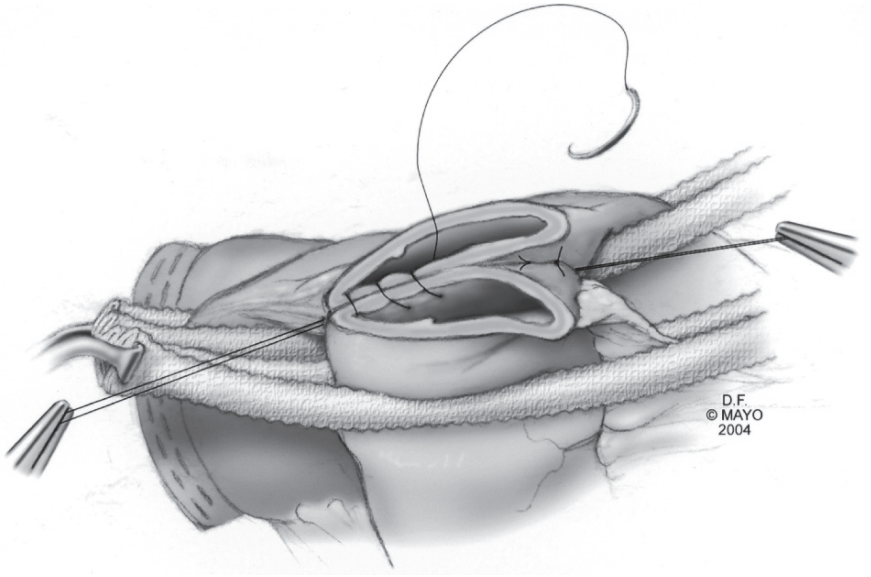

FIGURE 17.12. Anterior row of the inner running layer of a hand sewn anastomosis

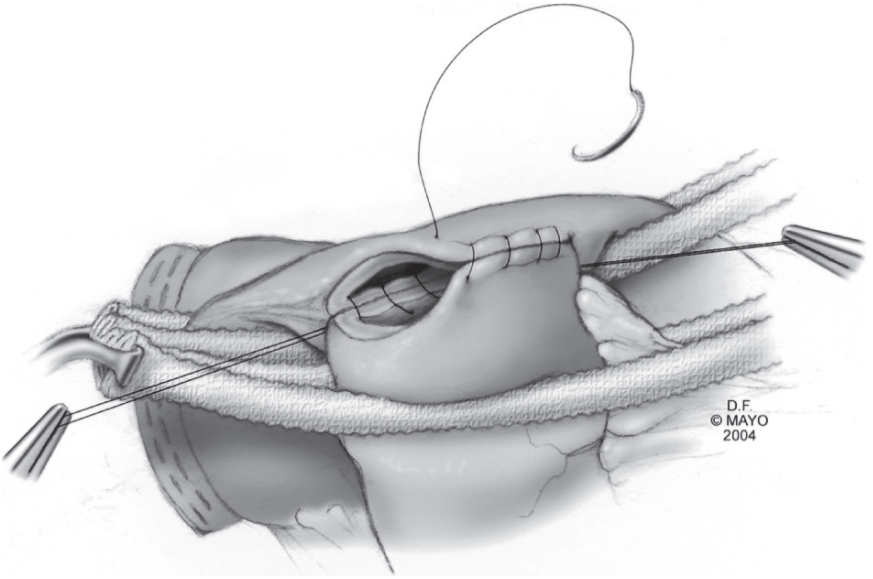

FIGURE 17.13. Anterior interrupted row of a hand sewn anastomosis

posterior aspect of the anastomosis. This suture is continued around the opposite corner to complete the anterior mucosal approximation. The suture is then tied to itself at the corner. The occluding bowel clamps are removed from the bowel to allow blood flow to return to the ends of the bowel. The final step includes the anterior second layer of 3–0 Lembert sutures approximating the serosal layer and thus bolstering the anastomotic line.

The second type of anastomosis utilized would be that of the stapled functional end-to-end anastomosis. This anastomosis

utilizes two firings of a linear cutting stapler (Meagher and Wolff, 1994). This anastomotic technique begins with the clearing the colon or terminal ileum of mesenteric fat for approximately two centimeters. On the specimen side of these cleared areas, l-cm transverse incisions are made on the antimesenteric borders of the bowel (Figures 17.14–17.17). Placing one of the two sides of the linear cutting stapler into each of the holes in the small bowel first, and then the colon, the stapler is gently closed approximating the small bowel and the colon along the antimesenteric border. Assuring that the mesentery is clear and the stapler is in good position it is fired and then removed. Upon doing this the previously separate ileal and colonic enterotomies become joined into a single enterotomy, and a pair of Babcock clamps are used to grasp opposite borders of this enterotomy at the anterior and posterior staple lines. A reloaded, long (100 mm) linear cutting stapler is then placed across the ileum and transverse colon, at a right angle to the previous staple line. By retracting the previous enterotomy, the stapler is fired completing the surgical resection and anastomosis. It is our practice to imbricate the corners and crossing staple line of our anastomosis with four 3–0 sutures. One is placed at the distal end of the longitudinal (first) staple line between the two joined segments of bowel to add mechanical strength to this end of the anastomosis. Two sutures are placed to invert each end of the transverse (second) staple line. Finally, one inverting suture is placed at the point where the two staple lines intersect. The mesenteric defect can be closed or left open depending on surgeon preference. Omentum, if available, can be placed over the anastomosis to provide further protection against postoperative anastomotic leak.

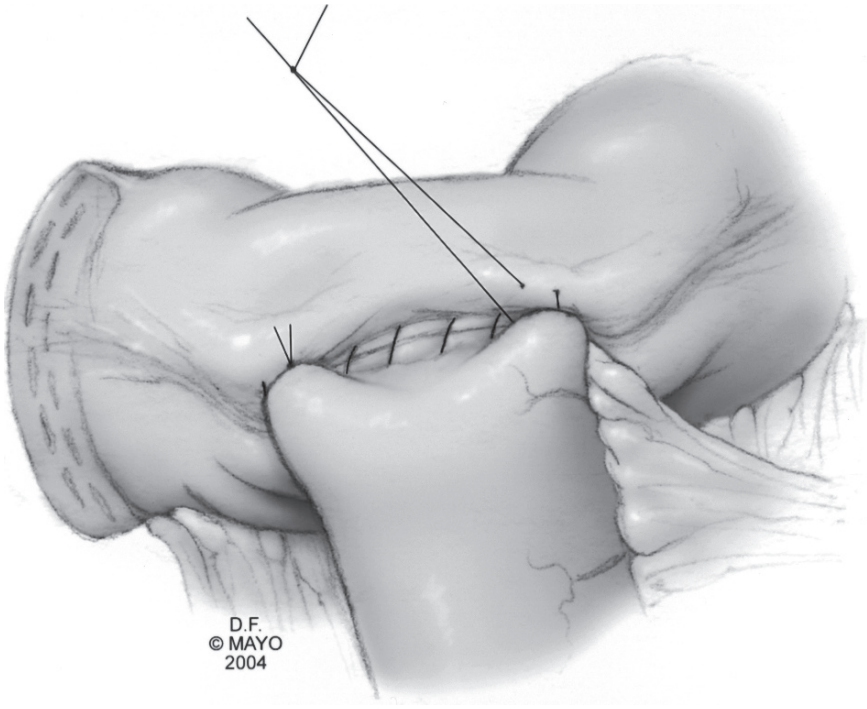

FIGURE 17.14. Incision in the specimen side of the large and small bowel

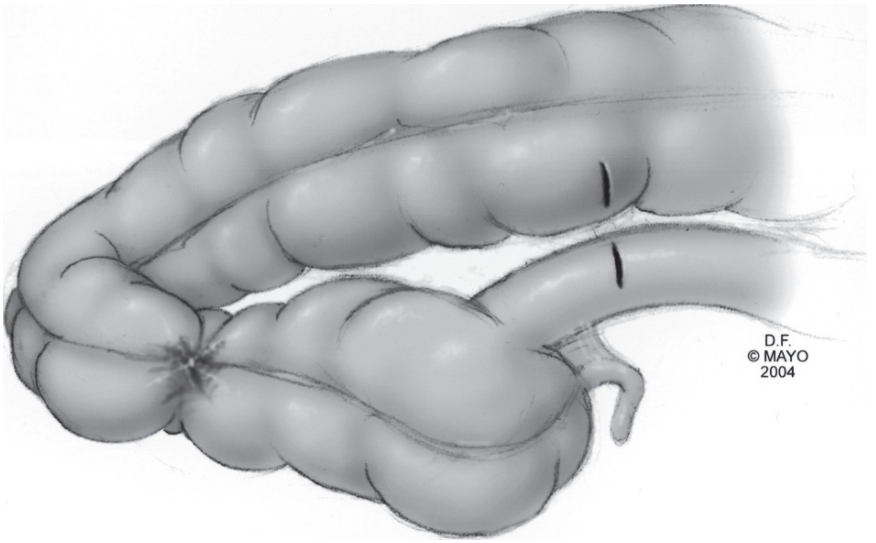

FIGURE 17.15. A 100 mm stapler passing down the remaining large and small bowel

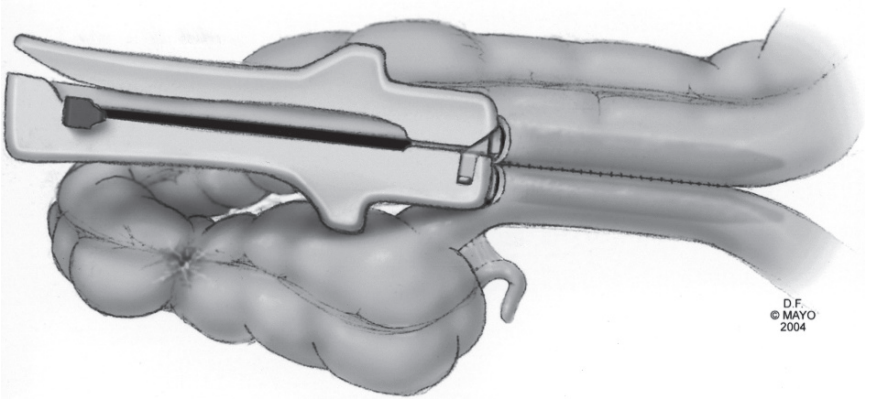

FIGURE 17.16. A 100 mm stapler dividing the specimen from the remaining bowel and completing the anastomosis

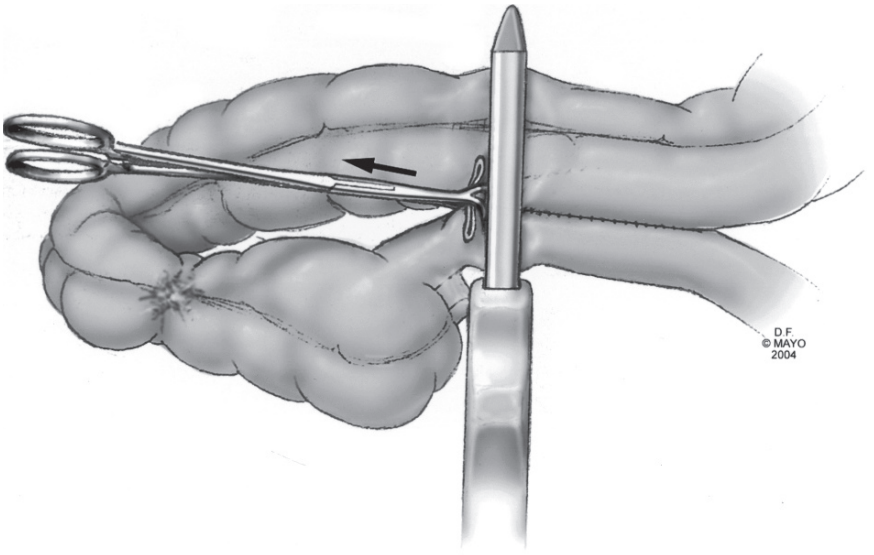

FIGURE 17.17. Imbricating the crossing staple lines with interrupted suture

Left or Sigmoid Colectomy

Resection of the left colon essentially retains the principles of the right colon. The initial approach to positioning is altered by placing the patient in the modified lithotomy position. We also use an additional fourth 5 mm trocar which is placed in the right lower quadrant, and typically utilize a medial to lateral approach, although a lateral to medial approach can be used successfully. The modified lithotomy position (legs-up) aids the surgeon with both abdominal and perineal exposure as well as stapler deployment. It is important to keep the thighs level with the abdomen when positioning given that elevation of the patients legs will interfere with instrument manipulation.

Once the trocars are in place (Figure 17.18) the medial approach may begin with the goal of vascular control being the first order of business. The more traditional lateral to medial approach may also be used in which vessel ligation would be left to the last step after complete mobilization of the bowel. Over the last several years I have come to prefer the medial to lateral approach although the choice should be left to surgeon preference.

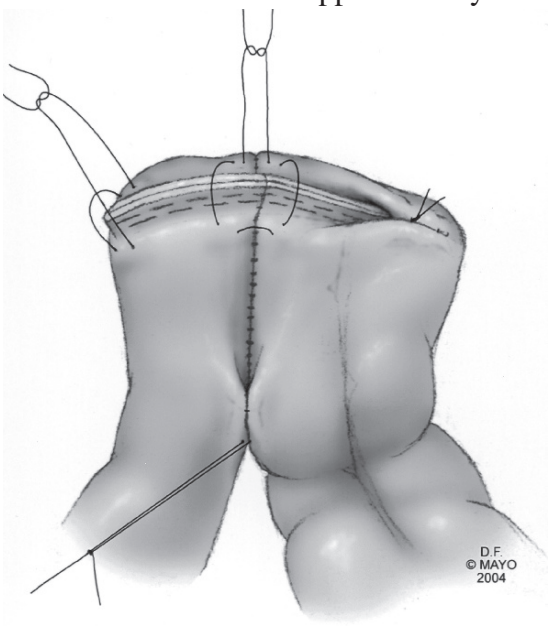

FIGURE 17.18. Port position and exteriorization incision for left sided operations or subtotal or total colectomies

The first step in the medial approach includes the dissection of the presacral space which is entered by first developing the presacral plane between the rectum and the patient's right pelvic side wall. The surgeon stands on the patient's right side and uses the suprapubic port and the right lateral port to complete this approach. The first assistant retracts the rectum and sigmoid with a Babcock placed through the left lateral port. With the sigmoid colon retracted cephalad and to the patient's left, the presacral window on the patient's right can be opened. Often traction is required on both the mid rectum and the superior rectal artery and vein complex in order to open this space. The objective of this approach is to open the presacral space and ultimately expose the left lateral sidewall and structures of the pelvic brim on the left (Figure 17.19). During this the left iliac vessels, presacral nerves, and left ureter can be easily identified and protected. Once these structures are identified the retroperitoneal space above the pelvic brim is opened with blunt dissection and electrocautery until the inferior mesenteric artery has been lifted off the retroperitoneum and the root is exposed at the level of the aortic insertion. Typically we will use electrocautery and the ligasure™ to accomplish this. Once this has been achieved, the IMA is placed on traction by the first assistant and the base is divided with a stapler or ligasure™ by the surgeon. This portion of the dissection can be the most difficult part of the procedure.

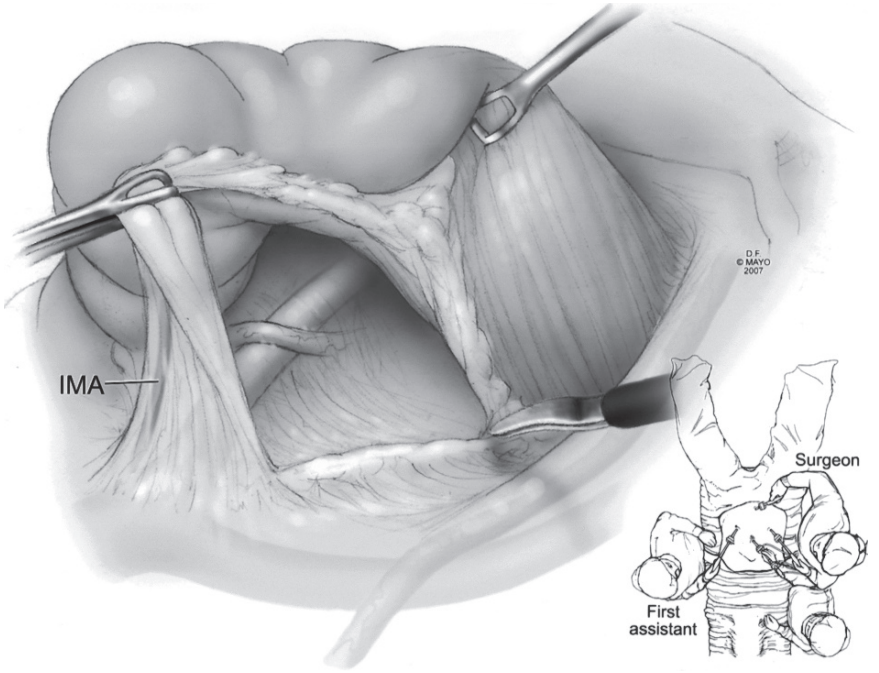

FIGURE 17.19. Surgeon positioning for a medial to lateral approach of the upper rectum and sigmoid. The presacral space has been opened with exposure of the left pelvic sidewall and exposure of the base of the IMA

Once the IMA has been divided, the left colon can be easily separated from the retroperitoneum with blunt dissection. This is a critical step to separate all of the retroperitoneal structures such as the ureter and gonadal vessels from the colon itself. Looking laterally from this medial position one can typically identify the "white line of Toldt" under the descending colon which aids the surgeon's ability to remain in the proper plane. If needed the medial dissection can continue up along the aorta to the root of the IMV which appears just to the patients left of the ligament of Treitz. If length is an issue the IMV may be taken as well and will typically allow for greater mobilization and a tension free anastomosis of any distal anastomosis. Completing this medial dissection results in only a thin lateral attachment to the side wall between the descending and sigmoid colon which requires further surgical dissection (Figure 17.20). This lateral attachment can now be taken quickly up the left side of the abdomen with the use of electrocautery. Of course in the more traditional approach this part of the dissection would be performed first, leaving dissection and ligation of the IMA or IMV to the last step.

With both lateral and medial approaches the splenic flexure is mobilized with a combination of lateral to medial dissection of the descending colon and elevation of the omentum off the distal transverse colon or greater curve of the stomach. If the omentum is to be preserved the first assistant, on the right, applies counter traction to the omentum by elevating it anteriorly**,** as the surgeon, who stands between

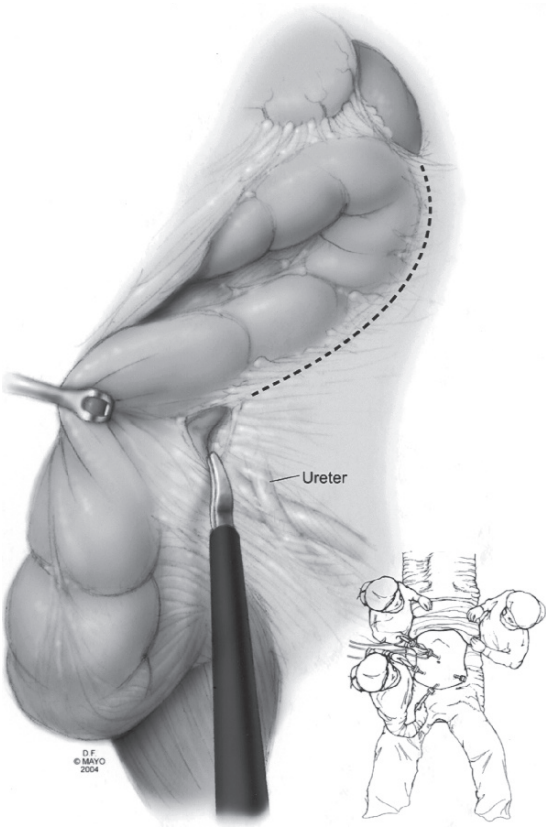

FIGURE 17.20. Lateral to medial dissection of the left colon

the patient's legs, separates it from the colonic border**,** thus entering the lesser sac (Figure 17.21). The omentum does not need to be mobilized any more than is necessary to drop the splenic flexure to the level of the umbilicus.

The final step includes division of the rectum and mesorectum which is achieved by dissecting from side to side along the mesorectum until one has reached the rectal wall utilizing electrocautery or ligasure™ (right to left). Upon completion of this the rectum is divided using a linear cutting stapler (Figure 17.22). Often I will utilize a rectal dilator to assess the length and size of the remaining rectum before dividing the rectum. These dilators are the length of a circular stapling devise and assure the surgeon that the circular stapler will reach the end of the rectal stump after division.

Once you have confirmed that there is sufficient mobility of the colon and that a tension free anastomosis is possible, the

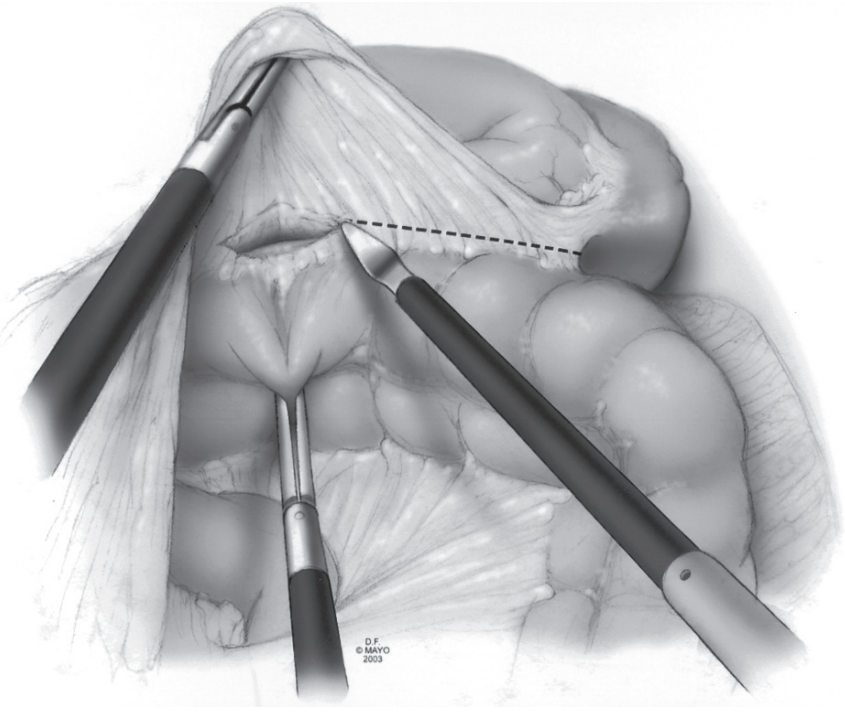

FIGURE 17.21. Elevation of the greater omentum off the distal transverse colon and entry into the lesser sac

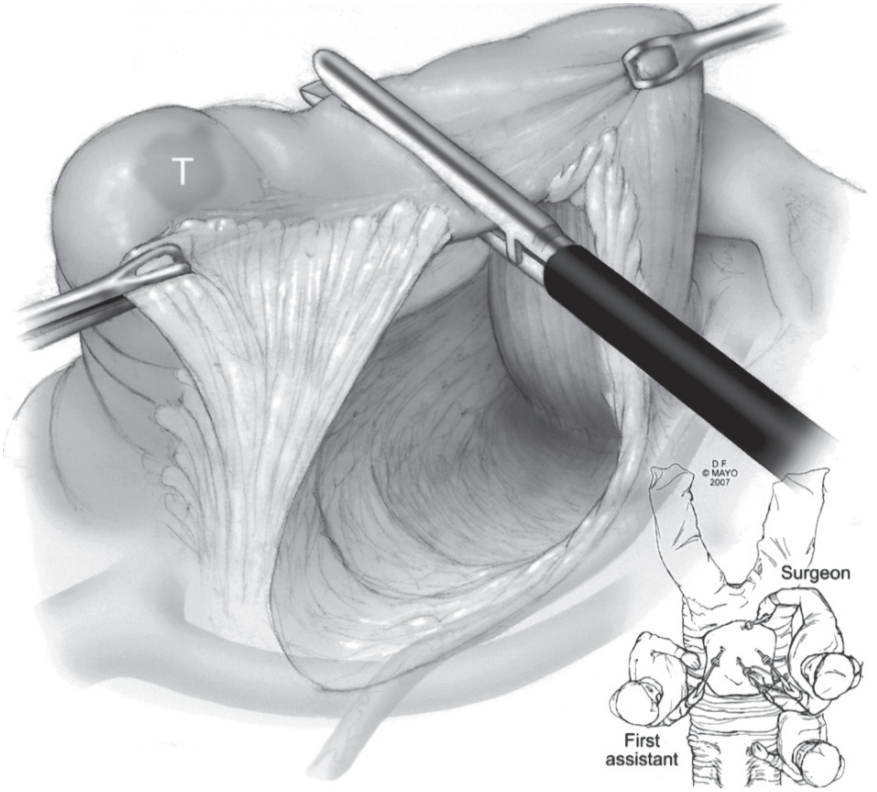

FIGURE 17.22. Division of the upper rectum with a laparoscopic stapler

specimen is ready to be removed. The sigmoid is then delivered through either a small incision in the midline similar to the right hemicolectomy, or alternatively through a small low midline or Pfannenstiel incision. We always use a wound protecting devise for the purpose of protecting the abdomen wall from either infection or oncologic concerns. The specimen is then delivered and the proximal margin identified. The bowel is typically cleared of mesentery and then is resected proximal to the tumor location.

After placing an anvil in the proximal bowel it is returned to the abdomen, and the fascia is closed or the wound protecting devise is simply twisted closed to recreate an air tight seal. The abdomen is re-insufflated, and the anvil is attached to the shaft of the stapling device, which has been introduced through the anus and advanced across the staple line (Figure 17.23). The stapling device is closed, the bowel ends approximated, and the stapler fired. A proctoscope is then used to examine the anastomosis for hemostasis and integrity. The cannulas are all removed under direct visualization and the fascial defects and skin closed.

In conclusion, the use of laparoscopic techniques is now available for nearly all diagnosis in the realm of colorectal surgery. The advantages of less pain, better cosmesis, fewer adhesions, faster recovery and hospital discharge are likely to expand the use of this approach. The important aspects which all surgeons must adhere to remain proper technique, oncologic staging

FIGURE 17.23. Stapled anastomosis of the descending colon to the upper rectum

and meticulous laparoscopic dissection. The case for the laparoscopic approach to colon cancer has been clearly defined by level one evidence, yet requires a well trained surgeon to implement these defined benchmarks.

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Sentinel Node-Based Immunotherapy of Colon Cancer

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INTRODUCTION

In spite of newly developed chemotherapeutic regiments, surgery, radiotherapy and the introduction of anti-angiogenic drugs the prognosis in disseminated colorectal cancer is poor. Recent investigations correlating expression markers with clinical outcome in patients with colon cancer indicate that tumor infiltrating lymphocytes are prognostically favourable, suggesting an important role for immune cells in the defense against cancer. Hitherto undertaken adoptive immunotherapy programs have focused on using tumor infiltrating lymphocytes with some success, but these cells are frequently suppressed by immune escape mechanisms induced by the tumor. However, recent advances in surgery and basic immunology have allowed the identification of the natural immune response, harbored in tumor draining lymph nodes, sentinel nodes. The sentinel node offers a promising new source, rich in tumor recognizing T lymphocytes that seem to be useful for immunotherapy. This chapter discusses relative immunology and rationale for using sentinel node acquired lymphocytes for adaptive immunotherapy of colon cancer.

TUMOR IMMUNE SURVEILLANCE

Historical Perspective

The idea of an immune surveillance mechanism against cancer was proposed long time ago by Paul Ehrlich. It was reformulated over half a century later by Burnet (1970), based on the increasing insights into the cellular mechanisms of immunity and transplantation. The concept states that lymphocytes continuously recognize and eliminate malignantly transformed cells; thereby, preventing the development of established cancer. The theory was questioned for several decades because studies of the immunodeficient mice models available at the time did not show any increased tumor susceptibility compared to normal mice. However, with the advent of molecular biology techniques, knock out mice lacking specific components of the immune system have provided strong experimental support. For example, $RAG2^{-/-}$ mice, which lack all T and B lymphocytes and mice unable to mediate IFN-γ signalling are also more sensitive to chemical carcinogens than wild type mice. Moreover, by 15 months of age; equivalent to senior status in mice, they also

displayed increased incidence of spontaneous tumors, mainly adenocarcinomas of colon, lung, and breast (Shankaran *et al*., 2001). These and other similar mouse models have provided important insight into the individual effector mechanisms contributing to immune surveillance in mice. In humans, such detailed investigations are not possible for obvious reasons. However, evidence in favor of tumor immune surveillance has accumulated.

Immunodeficient patients have increased risk for malignancies, primarily due to virally induced carcinogenesis, which does not give any unequivocal support to the immune surveillance hypothesis. However, in a retrospective analysis of renal transplant recipients, who received immunosuppressive drugs, an increased incidence for tumors of nonviral origin was reported (Birkeland *et al*., 1995). Although a direct effect of the medication cannot be excluded, the findings suggest a protective role of the immune system against human cancer development. Furthermore, a correlation between tumor-infiltrating lymphocytes and favourable prognosis has been described in several human tumors. The most compelling evidence for the existence of tumor immune surveillance in humans are reports of cancer patients who have developed immune responses against their tumors, especially if accompanied with spontaneous regression of the tumor (Mackensen *et al*., 1994). The first human tumor antigen, MAGE-1, was characterized in malignant melanoma (van der Bruggen *et al*., 1991) and numerous tumor antigens have now been described in tumors of different origin.

Immune Surveillance in Colon Cancer

Evidence for functional immune surveillance, as outlined in the previous section, also applies to human colon cancer. In the early 1990s, the capacity of tumor infiltrating lymphocytes from colon cancer to secrete cytokines upon stimulation with the autologous tumor was described by Hom *et al*. (1993). Moreover, lymphocytes reactive to colon cancer antigens have been detected in peripheral blood of colon cancer patients (Nagorsen *et al*., 2000).

As in malignant melanoma, tumor-infiltrating lymphocytes are associated with a more favorable prognosis. An impressive in-depth analysis, made by Pagés *et al*. (2005) of infiltrating immune cells on a cohort of 959 colorectal cancer specimens confirmed these findings. By using a combination of flow cytometry, immunohistochemistry, and quantitative real-time PCR, they demonstrated that tumors showing signs of early metastases have less infiltrating effector memory T lymphocytes, predominantly of the $CD8^+$ subset, than tumors without histological evidence for early invasion. Importantly, the immune cell infiltrate consisted of both CD4-positive (helper) and CD8-positive (cytotoxic) T lymphocytes and evidence of a so-called Th1 response, with expression of the cytokine IFN-γ, preferentially detected in tumors without signs of early invasion.

Tumor Antigens in Colon Cancer

Compared to malignant melanoma that is well-recognized as an immunogenic tumor, there is a paucity of tumor antigens in colon cancer which have been reported to elicit immune responses in patients.

Tumor antigens are usually grouped into four different categories: (1) Cancer-testis antigens: as the name implies, these proteins are expressed in the testis and are then reexpressed in tumors of different origin.

MAGE-1, mentioned above, belongs to this large family of tumor antigens. These antigens are attractive as targets in immunotherapy due to their absence from healthy adult tissues, with the exception of the testes. (2) Differentiation antigens: Proteins shared between tumors and the normal tissue from which the tumor has developed. (3) Widely occurring, overexpressed tumor-associated antigens: These proteins are not generally present in normal tissues, but they are detected in a wide variety of tumors of different origins. (4) Unique and shared tumor-specific antigens: antigens that have arisen due to point-mutations in tissue proteins.

On the basis of their cancer-testis antigen expression, tumors can be divided into high, intermediate, or low expressing. Whereas, for example, malignant melanoma and bladder cancer are considered high expressing tumors, with more than half of the known cancer-testis antigens expressed in 20–70% of tumors, colon cancers belong to the low expressing group. When interpreting these data, it is noteworthy that most studies addressing the cancer-testis antigen status of a certain tumor type have only investigated mRNA levels, which do not necessarily correspond to actual protein expression. Nevertheless, Li *et al*. (2005) have analyzed 121 colorectal cancers specimens with RT-PCR for expression of ten cancer-testis antigens, including NY-ESO-1, LAGE-1, MAGE-1, MAGE-3, MAGE-4, CT-10, SCP-1, SSX-1, SSX-2, and SSX-4. In this study, expression frequencies ranged from 1.7% to 27.3% with the majority below 10%. The most prevalent antigens were MAGE-3 and -4, which were detected in 22.3% and 27.3%, respectively, but 43.8% of tumors were negative for all of the investigated antigens. Twelve of the patients (9.9%) had NY-ESO-1-positive tumors, one of which also had antibodies against NY-ESO-1 in the serum. None of the remaining antigens were investigated with respect to immune responses, nor was their expression confirmed at the protein level. Thus, the cancer-testis antigens are sporadically expressed in colon cancers and may give rise to immune responses in a fraction of these patients. At least against NY-ESO-1 both B and T cell immunity responses exist. However, the low expression frequencies reduce their value as target antigens in immunotherapy. The differentiation antigens carcinoembryonic antigen (CEA) and Ep-Cam elicit spontaneous immune responses in colon cancer patients (Nagorsen *et al*., 2000). For these two antigens, both MHC class I and class II epitopes have been described, including antibody responses. To date, CEA is the most employed target antigen in active, specific immunotherapy trials.

Responses against a limited number of tumor-associated antigens have been detected in colon cancer patients. One of the most frequent genetic alterations in human cancers are mutations in the p53 suppressor gene. Because its role in colon cancer tumorigenesis is well-recognized, p53 has also been thoroughly investigated as a tumor antigen in its wild-type form. Both MHC class I and class II-restricted T cell responses, as well as an antibody response have been described. Importantly, a proliferative response against wild-type p53 was detected in peripheral blood of surgically resected colon cancer patients several years after the operation. However, the majority of p53-specific $CD4^+$ T helper cells failed to secrete effector cytokines, such as IFN-γ upon *in vitro* stimulation,

suggesting a suboptimal activation of these clones (van der Burg *et al*., 2003). In this study, patients with a detectable IFNγ-response against wt-p53 had higher numbers of tumor-infiltrating lymphocytes. This was interpreted as a support for p53-specific immunotherapy. Both mutated and wild-type p53 have been used in vaccination trials in colon cancer patients. Another tumor-associated antigen is Her2/neu, mostly studied in the context of breast and ovarian cancers. It is also expressed in a subset of colon cancers and a spontaneous T cell response in peripheral blood was reported (Nagorsen *et al*., 2000). On the other hand, there are conflicting data regarding the prevalence of Her2/neu overexpression in colon cancers, and it has not yet been explored as a vaccination target in this setting.

Ito *et al*. (2000) have identified T cell responses against several tumor-associated antigens in tumor-infiltrating lymphocytes and/or peripheral blood from patients, including SART-3, which is ubiquitously expressed in both normal and malignant tissues. SART-3 has been used in a phase I vaccination trial, and antibodies against the protein were detected following vaccination, but the antigens are otherwise unexplored in immunotherapy. The enzyme telomerase, which maintains chromosome length and allows cells to divide indefinitely, is widely expressed in tumors of different origin. Titu *et al*. (2004) have detected IFN-γ secretion by ELISPOT in peripheral blood leukocytes from a subset of colon cancer patients upon stimulation with two different MHC class I peptides derived from this protein, thereby adding telomerase to the list of potential tumor-associated antigens in colon cancer.

The fourth category comprises antigens resulting from mutations of normal genes. The antigens can, therefore be considered tumor-specific. Some of these mutated proteins are associated with the malignant transformation and have implications for tumor growth and/or patient survival. This property makes them one of the most promising targets for active immunotherapy. Unfortunately, very few antigens in this category have been identified in colon cancer. The tumor growth factor-beta receptor type II (TGFβRII) has received more attention. The human gene for this protein contains a polyA sequence which frequently is mutated in the subset of colon cancer with microsatellite instability (MSI). Saeterdal *et al*. (2001) have identified a frame-shift-mutation derived peptide which is recognized in the context of MHC class II by tumor-infiltrating lymphocytes from patients with MSI tumors. An actively processed MHC class I epitope, derived from the same region of the protein, has been described. Because these mutations may contribute to tumorigenesis, the TGFβRII is an appealing target for immunotherapy of MSI colon cancers.

The antigens mentioned above have been reported to elicit spontaneous T cell responses in colon cancer patients. A number of additional antigens have been used in vaccination trials in colon cancer; these are briefly discussed in the second section of this chapter. Furthermore, several antigens have been detected by screening of cDNA expression libraries derived from the autologous tumor with the patient's serum, termed SEREX. The expression pattern and the existence of a corresponding T cell response must be evaluated for each of these proteins, before they may qualify as promising tumor antigens, and they are, therefore, not discussed in this text.

Immune Escape Mechanisms

Obviously, the outgrowth of a malignant tumor is a failure of immune surveillance mechanisms. It has become increasingly clear that malignant tumors have developed a vast array of mechanisms allowing them to avoid detection and/or elimination. The term "immunoediting" was coined by Dunn *et al*. (2004) and described the interplay between the tumor and the immune system. Colon cancer has been regarded as an immunologically inert tumor compared to malignant melanoma or renal cancer. Not surprisingly, many tumor escape mechanisms have been described in the setting of colon cancer. For instance, an observation of peripheral blood leukocytes from colon cancer patients display reduced cytokine secretion upon stimulation (Heriot *et al*., 2000) which was normalized following surgical resection of the primary tumor. Colon cancers have been reported to express indoleamine-2,3-dioxygenase (IDO), a tryptophan-degrading enzyme, which may hamper T cell proliferative responses by depleting the tumor microenvironment from tryptophan (Uyttenhove *et al*., 2003). This likely reflects the immunosuppressive microenvironment created by the tumor; infiltrating lymphocytes from colon cancers result in reduced levels of the CD3ζ chain, which participates in the signal transduction cascade from the T cell receptor (Nakagomi *et al*., 1993).

A significant fraction of colorectal tumors display reduced expression or total loss of MHC class I molecules, thereby avoiding recognition by T cells. However, total loss of MHC makes a cell susceptible to lysis by Natural Killer (NK) cells. In a recent retrospective study, Watson *et al*. (2006) investigated 455 resected colon cancers with immunohistochemistry. MHC class I expression could independently predict disease-free survival, with high expression (76.5% of the samples) being the most favorable phenotype, followed by total absence of MHC molecules (9.9%), while tumors with low, but still detectable MHC expression (13.6%) had the poorest prognoses. However, Sandel *et al*. (2005) found very few infiltrating NK cells in specimen of colorectal cancer tissue, nor did they find any correlation with MHC class I expression. Thus, the role of NK cells in the immune surveillance of colon cancer mandates further investigation. Alternatively, tumor cells may escape tumor antigen-specific T cells by ceasing to express the tumor antigen. Khong *et al*. (2004) investigated tumor escape in a patient with malignant melanoma who first responded to immunotherapy, but later the disease progressed. In this case, one metastatic lesion had lost MHC class I expression while another concurrent lesion showed loss of tumor antigen expression. In immunotherapy, a way to circumvent the selection of antigen loss variants is to use tumor antigens crucial for tumor growth and/or survival, even though this further limits the number of available tumor antigens.

Naturally-induced regulatory T cells, defined by expression of the transcription factor foxP3, have during the last decade been recognized as a strong modulator of immune responses, both to foreign and endogenous antigens. The number of circulating foxP3-expressing regulatory T cells are elevated in cancer patients with a variety of tumors, including colon cancer (Wolf *et al*., 2003). This cell population has been associated with poor outcome in other malignancies but its role in colon cancer is not yet defined.

Central Tolerance

One concern that has been raised in regard to the unmutated self-antigens as potential targets in tumor immunotherapy is the possible existence of central tolerance against these proteins (Kyewski and Klein, 2006). This mechanism by which developing thymocytes with high avidity for self antigens are deleted early in their development, requires the expression of these antigens in the thymus. The self antigen-reactive cells are able to escape this selection process. They enter the peripheral circulation as mature, naïve T lymphocytes, and should consequently be of low avidity and therefore suboptimal for direct use in immunotherapy. Messenger RNA for several tumor antigens in the cancer-testis gene family has been detected in the thymus, including MAGE-A1, -A3, -A4, and NY-ESO. In addition, CEA, p53, and Her2/neu have been detected in normal, non-neoplastic human thymus at the protein level, preferentially in Hassal's corpuscles. In addition to central tolerance, the thymic expression of these antigens may contribute to the induction of foxp3-expressing regulatory T cells (Kyewski and Klein, 2006), a process which, interestingly, has been linked to Hassal's corpuscles (Watanabe *et al*., 2005). However, being expressed in the thymus does not exclude proteins from therapeutic targeting, as evidenced by MART-1 and tyrosinase, which have been detected in the thymus at the mRNA level. In spite of their expression in the thymus and the risk of negative selection of tumor responding T cell clones, they have served as targets in several immunotherapy trials in melanoma patients and some of them have been reported to be of clinical benefit for the patients. Immunotherapy of cancer in general and its use in the treatment of colon cancer in particular is addressed in the following section.

IMMUNOTHERAPY OF CANCER

The idea of using the immune system for treating cancer is 200 years old but translation of tumor immunosurveillance into clinical treatments has been made possible by the insights into cellular immunology and the molecular characteristics of tumor antigen recognition achieved during the last decades. Many different approaches have been made, both unspecific and more or less antigen-specific. Unspecific stimulation of the immune system include the use of Bacille-Calmette-Guérin (BCG) instillation in the urinary bladder for the treatment of bladder cancer and intravenous administration of cytokines. IFN-α may induce complete remission in hairy cell leukaemia, but the therapeutic effect of cytokine administration to patients with other malignancies is confined to occasional tumor regressions of malignant melanomas and renal cancer upon treatment with high-dose IL-2 or IFN-α. Thus, in the majority of cancer patients a more tumor-specific modality is necessary.

Active Immunotherapy – Vaccination

The first attempt of vaccination against cancer dates back to the late 18th century when Dr. Nooth, surgeon of the Duke of Kent injected himself with tumor cells from cancer patients. The use of whole tumor cell extracts, combined with adjuvant, has long been the dominating antigen preparation. However, since the characterization of tumor antigens in the beginning of the 1990s, it has become possible to use these proteins or

antigenic peptides derived from the antigens. More recently, the concept of DNA, and even RNA vaccination has been introduced. There are certain advantages and drawbacks with each antigen preparation.

The use of whole cell vaccines is limited by the availability of autologous tumors and because vaccines are designed for a single patient they are also limited by high costs. Although allogenic tumors or tumor cell lines have been used, there is a risk of stimulating an immune response against an irrelevant antigen. Vaccines targeting a single tumor antigen may be used for groups of patients, which reduce costs, but a potential drawback is the risk for immune escape through the selective outgrowth of tumor cells that have ceased to express the vaccine target. Compared to whole proteins, peptides are relatively easy to manufacture under good laboratory practice conditions, but the MHC haplotype of the patient must be taken into consideration. Even though whole proteins are more labour-intensive to produce they may give raise to a more diverse immune response. An interesting alternative is anti-idiotypic antibodies. These antibodies have an antigen-binding domain which mimics the surface of the antigen, and which may provoke an immune response against the tumor antigen.

Nucleic acids are easy and comparably inexpensive to produce in clinical grade and because the result is protein expression, it carries the advantages of whole protein vaccination. One drawback of injection of naked DNA is the risk of random integration into the genome. In this sense the use of mRNA for the antigen seems attractive. The immune response elicited by a vaccine is dependent on the presentation of the tumor antigen to T lymphocytes (described in more detail in the next section). An effort to improve this crucial step involves vaccination with antigen-presenting (dendritic) cells which have been preloaded with tumor cells, tumor antigen or transfected with cDNA from the autologous tumor.

Colon cancer vaccination trials, using most of the described approaches, have been undertaken and an extensive review of vaccination trials was recently published (Mosolits *et al*., 2005). A minor fraction of the trials has been randomized and controlled with the explicit aim of, and power to, assess therapeutic efficacy (phase III trials). Three sequential studies of an autologous whole tumor cell, admixed with BCG adjuvant (OncoVAX), have included a total of 704 patients with stage II and III colorectal cancer, randomized to either tumor cell vaccination after curative surgery or surgery alone. No statistically significant benefit was seen in stage III patients, but a considerable improvement in overall survival in the patients with stage II disease was evident upon intention-to-treat analyses. In the group of patients with stage II disease, the magnitude of the delayed type hypersensitivity skin reaction against autologous tumor cells, correlated with survival. Another autologous cell vaccine, administered with a viral adjuvant, was used in 310 patients with stage I-IV disease. These patients were compared to 257 patients receiving surgery alone. At 7 years follow-up, survival rates in the vaccinated group were significantly better than in the control group (43.4% versus 56.6%).

Tumor antigen-specific vaccination in colon cancer patients most often targets carcinoembryonic antigen (CEA), a membrane glycoprotein being over-expressed in more than 90% of colorectal tumors.

Vaccine, based on recombinant whole protein, was given to 24 resected colon cancer patients, together with granulocyte-macrophage colony-stimulating factor (GM-CSF). Follow-up revealed durable antibody and T cell responses which correlated with increased survival. A dendritic cell-based vaccine with cytokine-mobilized, autologous, antigen-presenting cells, loaded with a CEA-derived peptide, resulted in tumor regressions in 3/12 metastatic colon cancer patients. Again, the magnitude of the T-cell response appeared to reflect clinical outcome. Although the small number of patients in these studies must be kept in mind when evaluating the data, the results are promising. Furthermore, CeaVac, an anti-idiotypic antibody mimicking CEA, and gene-based vaccine with a poxviral vector encoding full-length CEA (ALVAC-CEA), have demonstrated their ability to stimulate antibody and T cell responses against CEA, and now await further evaluation.

Other tumor antigens that have been used in vaccination trials include Ep-Cam, p53, MAGE proteins, SART-3, and K-ras, all known to be capable of inducing spontaneous T cell responses in colon cancer patients. A number of additional molecules have also been targeted, such as the carbohydrate antigens MUC-1 and the closely associated Sialyl-Tn, the complementary regulatory protein CD55, human choriongonadotropin, and the oncofetal antigen 5T4. Another interesting vaccine candidate is the anti-apoptotic protein survivin, an anti-apoptotic protein against which tumor reactive lymphocytes from patients can be raised by *in vitro* stimulation. Moreover, it carries the possible advantage of being a survival factor for the tumor, thereby reducing the risk for immune escape. However, so far vaccination of stage IV disease patients has met with limited success and the same is true for most of these candidate antigens.

Most vaccination trials have been carried out in patients with an established tumor, often late stage disease. It is well recognized that the advanced cancer patient, in general, is immunosuppressed due to old age as well as to the presence of the tumor. As a result, there will be a compromised response to vaccinations. The setting of an established disease is also quite different from the situation with microbial vaccinations, which most often are prophylactic. The presence of large amounts of tumor cells, and the vast array of the immune escape mechanisms described above, have likely contributed to the relatively poor performance of therapeutic cancer vaccines to date. Methods for enhancing vaccine performance, such as prime-and-boost strategies with sequential administration of different antigen formulations, improved adjuvants, more carefully selected tumor antigens, and concomitant *in vivo* depletion of T regulatory cells, may improve the results. Nevertheless, some patients with late stage disease will, irrespective of the vaccination protocol, be unable to mount a productive immune response against their tumor. One way to circumvent the problem is passive immunotherapy, that is, adoptive transfer of tumor-reactive lymphocytes which have been expanded *in vitro*.

Passive, Specific Immunotherapy

In this context, both direct administration of antibodies and immunological effector cells are included. Two antibody-based therapies for colon cancer have recently been approved for patients with metastatic (stage IV) disease; one targeting the

epidermal growth factor receptor (cetuximab) and the other against the vascular endothelial growth factor (bevacizumab). The former is thought to exert its main therapeutic action by inhibiting growth factor signalling. The latter is anti-angiogenic. Antibody therapies, with the explicit goal of immunological eradication of cancer cells through opsonization and antibody dependent cytotoxicity, have not yet reached clinical application. A monoclonal antibody against Ep-Cam (17-1A) was administered to resected stage III patients and showed promising results in pilot trials. However, when tested in a large, randomized controlled study, including 2,671 stage III patients in 27 countries, no benefit was seen when used in addition to conventional chemotherapy, and survival was clearly inferior in the group receiving the antibodies as monotherapy (Mosolits *et al*., 2005).

The concept of adoptive, cellular immunotherapy was pioneered by Rosenberg *et al*. (1994) in malignant melanoma and involves isolation of tumor-reactive lymphocytes, mainly T cells, from the patient. The isolated T cells are expanded *in vitro* and then returned to the patient as an intravenous infusion together with intravenous infusions of interleukin-2; a major T cell growth factor. The source of tumor-reactive T cells was in this case tumor-infiltrating lymphocytes which were cultured for 4–8 weeks in the presence of IL-2 to levels of $> 10^{11}$ cells. Eighty six patients were treated according to this protocol and objective responses were seen in approximately one third of them. However, the lymphocytes persisted poorly after transfer and tumor progression most often ensued. By modifying the protocol, with the addition of lymphodepleting chemotherapy prior to transfer and use of a polyclonal T cell population consisting of both CD4-positive T helper cells and CD8-positive cytotoxic T cells, results have improved (Dudley *et al*., 2005). Objective responses have been noticed in 50% of patients, including occasional complete responders. This must be considered very promising in the context of metastatic disease with significant remaining tumor burden.

Adoptive Immunotherapy in Colon Cancer

Melanoma is the most studied and treated malignancy from an immunological point of view. This is partly due to its well-recognized immunogenicity. Another advantage for the immunologist is the superficial growth of tumor lesions, which makes the tumor-infiltrating lymphocytes relatively easily accessible. Colon-cancer infiltrating lymphocytes, expanded in IL-2, secrete a mixture of cytokines, including interferon-gamma and granulocyte/macrophage colony-stimulating factor and tumor necrosis factor alpha in response to the autologous tumor (Hom *et al*., 1993). However, they were unable to lyse autologous cancer cells *in vitro*, which contrasted to the expanded melanoma-infiltrating lymphocytes, which in the majority of cases display cytotoxicity against the autologous tumor (Yannelli *et al*., 1996). The reason for this cannot be determined from the studies referred to above, but possible explanations listed by Yannelli *et al*. (1996) include (1) overgrowth of T lymphocytes of other specificities than tumor antigens in the cultures, (2) the existence of repair systems in colon cancer cells rendering them relatively resistant to lysis, (3) absence of cytolytic cells among the tumor-infiltrating lymphocytes, or (4) absence of an immune

response against the tumor. Because accumulated evidence suggests that an immune-response against colon cancer does exist, the fourth explanation is unlikely. As discussed in the previous section, tumors generally develop strategies to circumvent the immune response. Evidently, these mechanisms as well as other aspects of tumor biology differ between melanoma and colon cancers. The findings from *in vitro* cultures did not deter Fabbri *et al*. (2000) from using tumor infiltrating lymphocytes, expanded in interleukin-2 to treat 22 patients with advanced cancers, 9 of whom had colon cancer. No clinical responses were detected in this setting. In a subsequent trial 39 patients who where classified as disease-free following metastasectomy were treated by the same protocol. Of 19 colon cancer patients, 8 remained disease free at evaluation after a median follow-up of 21 months.

Adoptive transfer of autologous activated macrophages has been attempted in advanced colon cancer patients. This cell population is derived from monocytes retrieved from patients' peripheral blood which are differentiated into macrophages *in vitro* by adding cytokines. A phase I/II trial conducted by Eymard *et al*. (1996) included 14 colon cancer patients with residual, bulky tumors. Apart from temporary disease-stabilization in three patients, no clinical benefit was seen.

Lymphokine-activated-killer (LAK) cells, being the patient's peripheral blood leukocytes *in vitro* activated with interleukin-2, have been used by other investigators. Dillman *et al*. (1991) combined LAK cell treatment with continuous interleukin-2 infusion in a comparatively large study of 117 patients with advanced tumors of different origin. In total, eight patients displayed clinical responses, but none was detected among the eight patients with colorectal cancer. Similarly, Hawkins *et al*. (1994) performed a phase II trial in metastatic or unresectable colorectal cancer, with interleukin-2 and LAK cells. Of 22 patients, 19 were able to complete the treatment protocol and one patient achieved a complete response, yielding a response rate of 5%. A slightly different approach was used by Soda *et al*. (1999) who treated 11 patients, including 7 with advanced colon cancer and significant remaining tumor burden, in a study using peripheral blood lymphocytes retrieved by leukapheresis. Before adoptive transfer, the cells were co-cultured with inactivated autologous tumor cells and cytokines to foster the outgrowth of tumor-reactive T cell clones. Each patient received five cycles of adoptive transfers and in three of the colon cancer patients at least one of the lymphocyte-tumor cell cultures resulted in cytotoxicity against autologous tumor cells. Interestingly, these patients also displayed disease stabilization and/or tumor regression, including a reduction in serum levels of carcinoembryonic antigen in one patient. No clinical responses were seen in patients without detectable cytotoxicity *in vitro*. Although limited by the small number of patients, the results may be regarded as promising when compared to the rather disappointing results with adoptive immunotherapy so far reported in colon cancer patients. The few adoptive transfer protocols with expanded lymphocytes from tumor-draining lymph nodes are not mentioned here, but are discussed in the fourth section of this chapter. First, the immunological properties of the lymph node are summarized, providing a rationale for using this cell population

as an alternative source of tumor-reactive lymphocytes.

THE IMMUNE RESPONSE IN THE LYMPH NODE

The T and B lymphocytes are the mediators of adaptive immunity. Following their maturation in primary lymphoid organs, thymus and bone marrow, respectively, they commence a continuous journey between the blood and the secondary lymphoid organs of lymph nodes and the spleen. While recognition of blood-born antigens mainly occurs in the spleen, the lymph nodes serve as collecting stations for antigens from peripheral tissues.

Lymph Node Anatomy

The lymphatic system, first described by Olof Rudbeck in the mid 17th century, consists of lymphatic vessels which collect extracellular fluid from the tissues. The vessels interconnect, becoming progressively larger until they coalesce into the thoracic duct, which empties its content into the left subcalvian vein. Lymph nodes are distributed along the lymphatics. They are bean-shaped structures, covered by an outer, fibrous capsule. Histologically, two main regions can be distinguished; the cortex and the medulla. The cortex can be further subdivided into an inner paracortex, which is the T cell-area of the node, and the more superficial B-cell areas of primary follicles and germinal centres. Lymph enters the node via multiple afferent lymphatic vessels and leaves through a single efferent lymphatic vessel at the lymph node hilus, which also harbors a lymph node artery and vein.

Antigen Recognition in the Lymph Node

Lymphocytes enter the lymph node from the blood. Lymphocyte transmigration through the wall of the high endothelial venules in the lymph node is a highly regulated process, guided by specific expression of cell-surface molecules on recirculating lymphocyte subsets. In the lymph node, T and B cells find their way to their respective compartments by means of chemotactic gradients of soluble molecules which, again, bind subset-specific cell-surface molecules. Here, lymphocytes encounter tissue-derived antigens, both foreign and self molecules, displayed by professional antigen-presenting cells (APC). Antigens are transported to the lymph node with the afferent lymph, either engulfed by an antigen-presenting cell in the tissue as soluble proteins or as soluble proteins, which are fagocytosed by a lymph node-resident APC (Itano and Jenkins, 2003). Because immune responses against tumors are mainly attributed to cellular immunity, the focus here is on the activation of T cells.

Dendritic cells (DCs) are the main antigen-presenting cells in the lymph node, which are capable of stimulating and activating naïve T cells (Itano and Jenkins, 2003). Bearing in mind that DCs not only engulf exogenous antigens but also participate in the clearance of apoptotic cells in peripheral tissues, it is evident that the contact between the DC and the T cell has to be strictly regulated to avoid T cell activation against self antigens. In its resting state, a DC is very efficient in phagocytosis, but when encountering inflammatory stimuli or other "danger signals", such as those evoked by binding of microbial products to innate pattern-recognition receptors, it undergoes a maturation process. During

this process, the DC converts from antigen collecting to antigen presentation, i.e., digesting phagocytosed proteins into peptides, which are displayed on the cell surface by MHC molecules. Here, the antigenic peptides are accessible for recognition by T cells through the T cell receptor (Banchereau *et al*., 2000). Importantly, an immature DC can also present antigens, albeit very inefficiently. In fact, antigen-presentation by an immature DC can even, under certain circumstances, be tolerizing to T cells. By contrast, a mature DC has an increased number of peptide-MHC complexes on its cell surface. Moreover, it has up-regulated costimulatory molecules which bind their respective ligands on the surface of the T cell. This costimulatory signal, delivered in conjunction with antigen recognition by the T cell receptor, is necessary for productive T cell activation.

Outcome of T Cell Activation

The clonal selection theory was suggested by McFarlane Burnet, to explain how the adaptive immune response is initiated by the recognition of non-self, stating that every lymphocyte bears a unique antigen receptor. When an antigen binds to the receptor with high enough affinity the lymphocyte is activated and proliferates to form a clone of identical cells. During T cell development in the thymus, the immature thymocyte starts to express a T cell receptor (TCR) and one of two different coreceptor molecules. Depending on coreceptor expression, T cells are classified into either $CD4^+$ T helper cells, which exert their function mainly by secretion of cytokines, or $CD8^+$ T cells, which may directly kill virus infected or, by other means, transformed cells. In contrast to B cells, which respond to soluble, intact proteins, the T cell recognizes antigens as peptide fragments bound to major histocompatibility (MHC) molecules on the surface of other cells. $CD8^+$ T cells bind to MHC class I molecules, which are expressed by all nucleated cells and mainly present peptides derived from intracellular proteins. $CD4^+$ T cells bind to MHC class II molecules, expressed by APCs, such as DCs, but also by macrophages and B cells, which are able to present peptides from internalized, extracellular proteins. However, as discussed in the previous section, DCs are considered the main activators also of $CD8^+$ T cells, by means of their superior antigen-presenting and costimulatory functions. This is accomplished through a mechanism called *cross-presentation*, by which phagocytosed extracellular proteins are presented on MHC class I molecules. Recent evidence indicates that a productive immune response is initiated by a DC which activates both a $CD4^+$ T cell and a $CD8^+$ T cell, thereby providing a cellular "bridge" for delivery of helper signals from the $CD4^+$ T cell to the $CD8^+$ T cell. (Castellino *et al*., 2006). This is important for the development of immunological memory (see below).

Following activation, the T cell undergoes clonal expansion (Figure 18.1) and the progeny differentiates into effector cells. Differentiating $CD8^+$ T cells become competent in killing target cells by release of cytotoxic proteins, whereas $CD4^+$ T helper cells acquire the ability to secrete effector cytokines. Depending on their cytokine pattern, murine T helper cells may be classified as type 1 (Th1) cells, secreting mainly IFN-γ, IL-12 and TNF-α, or type 2 (Th2) cells, secreting IL-4, IL-5, and IL-13. Albeit human T helper cells show certain heterogeneity with respect

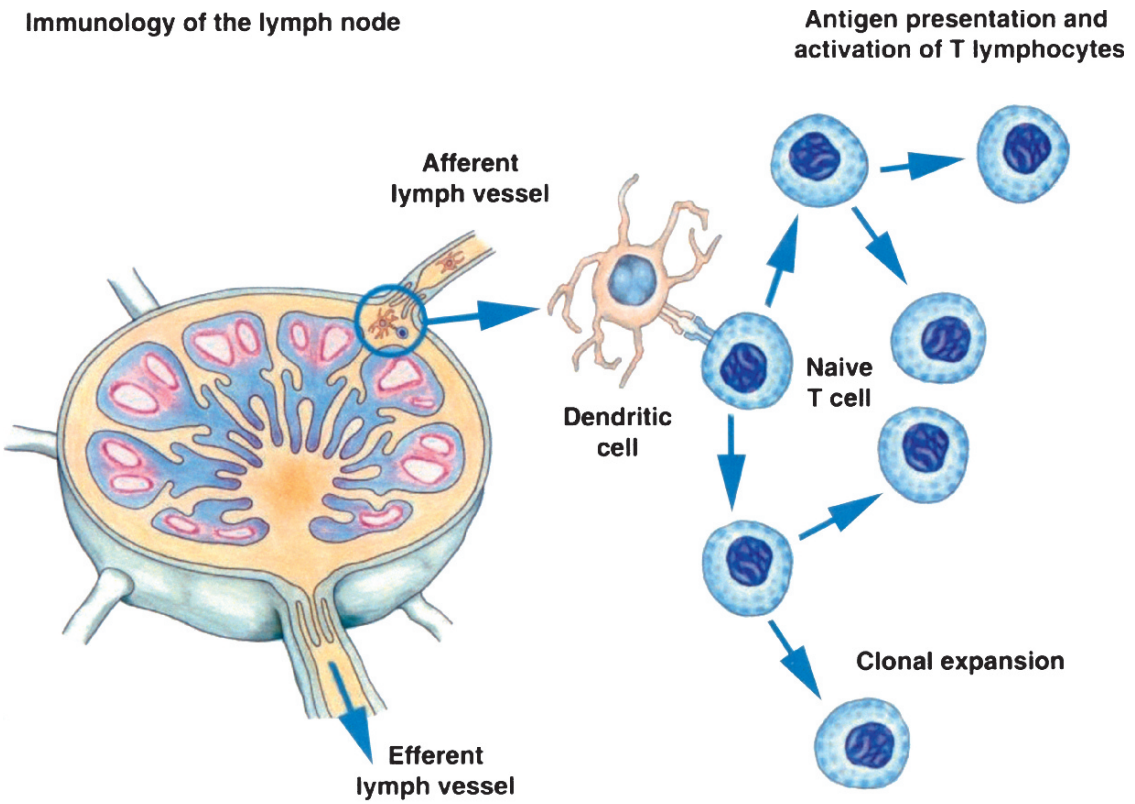

FIGURE 18.1. Immunology of the lymph node. Afferent lymph vessels contain draining lymphatic fluid with particles, antigens and antigen presenting cells. Material endocytosed by antigen presenting cells leads to activation and maturation towards a dendritic cell that presents antigenic peptides to naïve T lymphocytes. T lymphocytes with appropriate T cell receptors that have affinity for the class I peptide complex ($CD8^+$ cytotoxic T lymphocytes) presented by the dendritic cells or T lymphocytes recognizing peptides presented by the class II molecule ($CD4^+$ T helper cells) become activated. Activated T lymphocytes start to divide and undergo clonal expansion, a process that results in T effector lymphocytes that pass via the efferent lymph vessels and the thoracic duct before introduced to the circulation. In parallel, activation leads to the formation of long term memory T lymphocytes

to their cytokine secretion profiles, the Th1/Th2 paradigm has been useful for revealing fundamental aspects of T helper cell functions. Th1 cells promote cellular immunity, activating macrophages and providing costimulation to cytotoxic $CD8^+$ T cells, whereas Th2 cells act primarily on B cells, thereby augmenting antibody responses. The type 1 response is considered the most important effector mechanism in tumor immunity, and in human colon cancer patients the protective effects of a Th1 immune response have been confirmed (Pagés *et al.*, 2005).

Immunological Memory

Following antigen clearance, the majority of effector cells undergo apoptosis. However, antigen exposure usually leads to the development of immunological memory, which gives rise to a secondary response upon antigen reencounter, which is both faster and stronger than the primary response. Memory is mediated by

long-lived memory cells, which are the descendants of effector cells in the primary response. B cell memory segregates into two distinct cell types; small memory B cells, present in secondary lymphoid organs, and fully differentiated plasma cells capable of immediate antibody secretion, which reside in the bone marrow and in peripheral tissues. Also, in the case of T cells, two major subtypes of memory cells can be distinguished: central and effector memory cells. The former are characterised by their expression of the chemokine receptor CCR7 and reside in secondary lymphoid organs. They have limited immediate effector functions, but readily proliferate and differentiate into effector cells upon stimulation. The effector memory cells, by contrast, are capable of executing immediate effector functions upon antigen encounter, but have a limited capacity to divide. They can be distinguished from their central counterparts by their lack of the lymph node homing receptors CCR7 and CD62L. In peripheral blood, $CD4^+$ memory cells are predominantly of the central type, while the majority of $CD8^+$ memory cells are effector cells (Sallusto *et al*., 2004). In adoptive transfer studies of tumor-reactive $CD8^+$ memory cells in mice, the central memory cells seem to be more reliable in mediating efficient tumor regression (Klebanoff *et al*., 2005).

How are effector lymphocytes, from the primary immune response, rescued from apoptosis and how can they survive to become memory cells? These are questions of immense importance for understanding immunity in general, and in designing successful immunotherapies in particular. The $CD4^+$ T cell seems to be crucial, both to long-term B cell memory and $CD8^+$ T cell memory cell generation. Furthermore, without helper signals from a $CD4^+$ T cell during the primary immune response, $CD8^+$ T cell memory cells are unstable, exhibit reduced effector functions, and are prone to apoptosis (Janssen *et al*., 2005).

SENTINEL NODE ACQUIRED LYMPHOCYTES FOR IMMUNOTHERAPY

Sentinel Node Detection in Colon Cancer

Lymph nodes draining solid tumors are often the first sites for metastases to appear, an event of major prognostic, and hence, therapeutic importance. This is especially true for colorectal cancer, where the regional lymph node status is the major determinant of patient survival. For patients with localized disease (stage I–II) the 5-year-survival following curative resection is 80–90%. However, when the tumor has spread to the regional lymph nodes (stage III), the 5-year-survival is only 50–60% and in patients presenting with distant metastases (stage IV), it is <10%. Chemotherapy has not proven beneficial in stage II patients. In stage III, however, the combined treatment with 5-fluorouracil/folinic acid confers an absolute survival benefit of 10% and is considered standard therapy. In the setting of distant metastases, surgical resection of solitary liver metastases, as well as palliative chemotherapy, improve both survival and quality of life. With the advent of new chemotherapeutic agents, being added to the standard 5-fluorouracil/folinic acid combination, the prognosis has improved (Meyerhardt and Mayer, 2005). However, the median

survival in the Stage IV cases remains less than 2 years. Thus, there is a need for novel therapeutic approaches.

The sentinel node concept was originally formulated by Cabanas (1977) for penile carcinoma, stating that the lymphatic drainage from a tumor area arrives to a primary draining, or sentinel lymph node before proceeding to other lymph nodes. Therefore, the histopathological status of the sentinel node can be regarded as representative for the entire lymphatic field. By peritumoral injection of a tracer substance, either a radioactive compound or a blue dye, this node can be identified intraoperatively and subjected to detailed analysis. This concept is widely accepted in breast cancer and melanoma, where the histopathological status of the sentinel node has a large impact on the extent of surgery and postoperative treatment.

Sentinel node biopsy has also been applied in colon cancer (Dahl *et al*., 2005), although its role has not yet been fully established. Bowel resection due to colon cancer includes at least one of the major mesenteric vessels on each side of the tumor. Because sentinel nodes may be detected outside the regular resection margins, their localisation may influence the extent of the surgical procedure. In addition, accurate staging of a colonic tumor requires examination of a minimum of 13 resected lymph nodes by the pathologist. Sentinel node detection permits a more detailed examination of this particular node, including multi-level sectioning and immunohistochemstry. As a consequence, a significant fraction of patients otherwise diagnosed with node negative (stage I/II) disease are upstaged to stage III, thereby qualifying for post-operative, adjuvant treatment. Thus, sentinel node detection may improve survival in colon cancer.

Immune Responses in Sentinel Lymph Nodes

Although most investigators have focused upon the tumor-infiltrating lymphocytes, some studies have also addressed lymphocytes from the tumor-draining lymph nodes. There are several reports on immune suppression of tumor-draining lymph nodes. Most of these studies have been performed in malignant melanoma and, in some cases, breast cancer, due to the well-established role of sentinel node detection in these patients. Regarding tumors of other origin, most studies are based on regional lymph nodes, i.e., without sentinel node detection. An interesting study by O'Sullivan *et al*. (1996) compared proliferative responses upon mitogenic stimulation and cytotoxicity against an allogenic tumor cell line in tumor-draining lymph nodes with more distantly located nodes in 23 patients operated on due to esophageal squamous cellular cancer. They found that lymphocytes from tumor-draining lymph nodes in all patients showed decreased performance in both tests relative to control nodes. However, in patients with adenocarcinoma of the esophagus; the corresponding assay only detected immunosuppression in 2 of 26 cases, illustrating the divergent behaviour of different tumor types.

With respect to colon carcinoma, Pihl *et al*. (1976) have analyzed the reactivity of regional lymph nodes. Lymph node cytotoxicity against autologous tumor cells was detected in 32/142 cases with stage II–III tumors, excluding nodes with macroscopic tumor deposits. Immune reactivity was detected in 23% of the investigated lymph nodes located within 5 cm from the primary tumor, compared with 13% of the more distant nodes. This difference did not reach statistic significance. However,

since sentinel node detection in colon cancer has revealed draining lymph nodes located in the entire mesentery, sometimes near or even outside the conventional resection margins (Dahl *et al*., 2005), some of the reactive distant nodes in the study may have been sentinel nodes. The authors also noted that histological evidence of immune reactivity, namely sinus histiocytosis and hyperplasia of T and B lymphocyte populations, correlated with immunological reactivity.

Triozzi *et al*. (1994) adopted preoperative injection of a radio labelled monoclonal antibody against the tumor-associated mucin TAG-72 in patients with colon cancer, enabling intraoperative identification of lymph nodes containing shed tumor antigen and/or tumor cells by means of a hand-held gamma-detection probe. In all lymph nodes identified by the probe, these investigators found proliferative responses against autologous tumor cells. This was not the case for uninvolved lymph nodes.

Marits *et al*. (2006) have investigated sentinel-nodes in 15 patients with stage II–IV colon cancer. Lymphocytes from sentinel and non-sentinel nodes as well as tumor-infiltrating lymphocytes and peripheral blood leukocytes were assayed for immune reactivity against an autologous tumor extract with [^{3}H]-thymidine incorporation. In the eight patients with stage II disease, sentinel node acquired lymphocytes proliferated in response to the autologous tumor. In three of these patients, proliferative responses were also seen in non-sentinel nodes. In the patients with stage III–IV disease, i.e., in the presence of tumor cells in the sentinel lymph node, antigen-dependent proliferation was detected in one case of six. In this study, both tumor-infiltrating lymphocytes and peripheral blood leukocytes were unresponsive in the proliferation assays. In six of the patients, the secretory response to the Th1 cytokine IFN-γ was investigated and found to correlate with the results from the proliferation assays. (Marits *et al*., 2006). Lymph nodes draining metastases were detected in a similar way using tracer substances intraoperatively. Subsequent analyses showed tumor-reactive lymphocytes with a Th1 response located predominantely in these lymph nodes named "metinel nodes" (Dahl *et al*., 2007).

These four studies collectively indicate the presence of an immune response against human colon cancer in lymphocytes from tumor-draining lymph nodes. Because the lymphatic drainage pathways from a colonic tumor are specific for each patient (Dahl *et al*., 2005), which lymph nodes that are truly "tumor-draining" cannot be determined without specific sentinel node detection.

Lymph Node Based Treatments

Using the draining lymph node in cancer immunotherapy is a logical consequence of its central role in the initiation of an immune response and experimental evidence of an *in vivo* expanded population of tumor-reactive lymphocytes, not present in non-draining lymph nodes (Figure 18.2).

Building on results from an animal model of a poorly immunogenic, murine sarcoma, Chang *et al*. (2003) have conducted a Phase II clinical trial in patients with advanced renal cell carcinoma. Instead of relying on the primary tumor as immunogen, a tumor vaccine consisting of autologous tumor cells admixed with the bacterial adjuvant Bacille Calmette-Guérin (BCG) was used. One week after vaccination, lymph nodes draining the

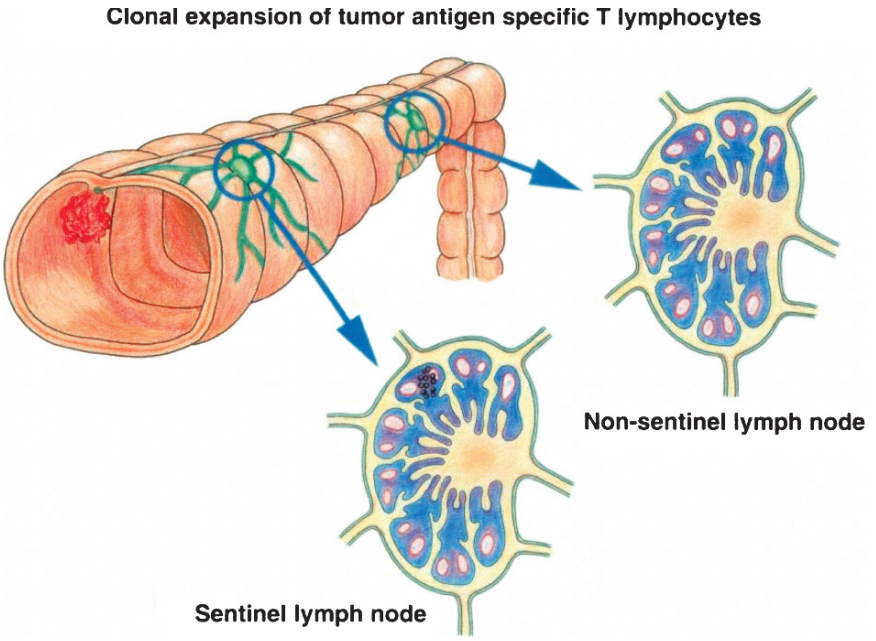

FIGURE 18.2. Clonal expansion of tumor antigen specific T lymphocytes. The growth of the colon cancer promotes the development of vessels including lymphatic vessels resulting in a connection with a lymph node. This tumor draining lymph node, the sentinel node, will receive tumor cells, debris and a transport of antigen presenting cells. The sentinel node antigen presenting cells will activate T lymphocytes leading to a clonal expansion, whereas non-sentinel nodes do not contain any expanded T lymphocyte populations with the ability to recognize tumor antigens. Thus, the sentinel node is the natural location for expansion of the tumor specific immune response

injection site (inguinal nodes) were removed and lymphocytes were expanded with a combination of anti-CD3 antibody and IL-2 for 6–8 days. Following this protocol, 100% of the cells were $CD3^+$, of which approximately 75% were $CD8^+$, and the remaining 25% were $CD4^+$ lymphocytes. All in all, 34 patients were treated with a mean of 3.9×10^{10} cells, resulting in five complete responses of varying duration and four partial responses (Chang *et al*., 2003). Similar approaches, i.e., adoptive transfer of *ex vivo* expanded lymphocytes from vaccine-primed lymph nodes, have been attempted by other investigators in malignant melanoma, head-and-neck cancer, and malignant glioma, though with less promising results.

Yano *et al*. (1999) used regional lymph nodes obtained during pulmonary lobectomy due to primary lung cancer to generate lymphokine activated killer cells (LAK). The lymph node cells were cultured for 14–16 days with high-dose IL-2 (2,000 U/ml) and given back to the patient, who also received multiple subcutaneous injections of IL-2. According to this protocol, nineteen patients were treated with an average of 7.4×10^9 cells. An increase in cytotoxic activity against an unspecific target cell line was detected in peripheral blood of the patients, but no survival benefit compared to surgery alone could be detected.

In colon cancer, detection of lymph nodes with the TAG-72 antibody (see the

previous section) was used to identify draining lymph nodes from patients with unresectable (stage IV) tumors (Kim *et al*., 1999). Lymph node cells were cultured *ex vivo* for 10–14 days, following an initial stimulation with anti-CD3 antibody and IL – 2 (100 CU/ml). Thirty-two patients were treated with infusion of a mean of 1.6 $\times 10^{10}$ expanded, predominantly $CD3^+$ cells, without addition of exogenous cytokines. Cells consisted of a mixture of $CD4^+$ and $CD8^+$ cells, displaying cell surface markers of activation and expressing the genes for IFN-γ, IL-4, IL-5, and GM-CSF. In two of the patients, 1×10^8 cells were [^{111}In]-labelled and co-administered with the rest of the expanded cells. However, the cells did not home to the tumor within the 96 h study period, but instead accumulated in bone marrow, lung, liver, and spleen. The therapy was well tolerated, with the exception of occasional fever and chills following the cell infusion. One patient displayed 80% reduction of a retroperitoneal tumor mass. Interestingly, the cells infused into this patient secreted the highest levels of IL-4 and GM-CSF, and an infiltration of macrophages was seen on a liver biopsy performed 19 months after the infusion. In addition, 4 patients had mixed or minor responses and 15 patients showed stable disease for 4–12 months, which rendered the study group a median survival of 12.5 months, compared to 5.8 months in historical controls. Although this difference does not reach statistical significance mainly due to the small study group, the results are promising considering the advanced stage of disease in the treated patients.

The sentinel node technique has, thus far, only been emulated in one immunotherapy trial (Karlsson *et al*., 2007 submitted). They expanded sentinel node-acquired lymphocytes *in vitro* using a combination of IL-2 and an autologous tumor extract (Figure 18.3). Sixteen colorectal cancer patients participated in the study, five of which had stage II tumors, two had lymph node positive (stage III) disease and the remaining nine patients presented with distant metastases. The expansion protocol with low-dose IL – 2 and intermittent restimulation with exogenous antigen resulted in a, predominantly $CD4^+$ T cell population, secreting IFN-γ in response to the tumor extract, i.e., a Th1 response. An average of 71 million cells were transfused back to the patients, with no apparent side effect or toxicity. In the nine patients with stage IV tumors, survival time was significantly increased in comparison to historical controls, and complete response with no remaining tumor growth was seen in four of the patients. Considering the advanced disease stage these results must be considered promising. Interestingly, experimental support for the efficacy of $CD4^+$ cells in adoptive immunotherapy was recently provided by Wang *et al*. (2007). Treating mice with established tumors with adoptive transfer of *in vitro* activated tumor draining lymph node cells, they demonstrated that $CD4^+$, as well as $CD8^+$ T cells, could have therapeutic efficacy on their own. However, administration of a combination of both subsets had synergistic effects, resulting in complete regression of tumors.

In conclusion, the studies by Kim *et al*. (1999) and Karlsson *et al*. (2007) provide evidence that adoptive immunotherapy of colon cancer is possible. By the advent of the sentinel node technique, reliable identification of truly tumor-draining lymph nodes has become possible, provid-

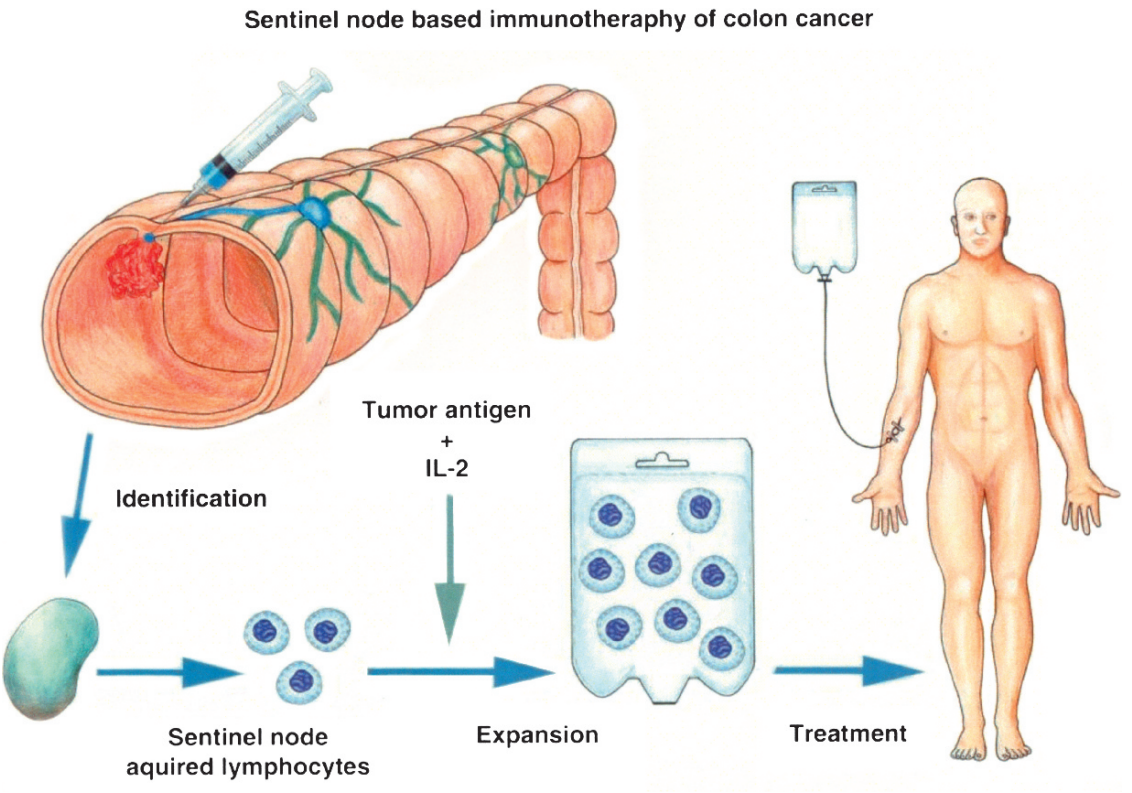

FIGURE 18.3. Sentinel node based immunotherapy of colon cancer. The sentinel node is identified by injecting the patent blue dye superficially around the tumor. Within minutes the lymphatic drainage will accumulate in a sentinel node, turning it blue. T lymphocytes are harvested from the sentinel node by making single cell suspensions for culture in vitro. The single cell suspension from the sentinel node contains T lymphocytes, B lymphocytes and antigen presenting cells. The T lymphocytes are activated by feeding the single cell suspension a tumor extract from the patient, an extract that is endocytosed, processed and displayed by the antigen presenting cells to T lymphocytes. To support activation and clonal expansion of tumor recognizing T lymphocytes the T cell growth factor IL-2 is included in the cultures. By repeated rounds of activation and stimulation, tumor antigen recognizing T lymphocytes overcome immunosuppresion, induced by tumor produced factors, and become clonally expanded. After 4 weeks in culture clonally expanded T lymphocytes are reintroduced back to the patients by means of a regular transfusion. T effector lymphocytes will now seek areas of inflammation containing metastatic tumor cells, and T memory cells will patrol and hibernate waiting for the appropriate time for reactivation in a state of vaccination

ing a promising cellular source for *in vitro* expansion. Using clinical trials to gain further knowledge of the mechanisms leading to tumor regression in experimental models, a cure, even for patients with advanced disease, may become a reality in the near future.

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A. Diagnosis

19
Rectal Cancer: Preoperative Staging Using Endorectal Ultrasonography (Methodology)

Ho-Kyung Chun, Yong Beom Cho, and Soon Jin Lee

INTRODUCTION

The prognosis of patients with rectal cancer is closely related to accurately assessing the extent of tumor within or beyond the rectal wall, and to the presence or absence of lymph node involvement. The risk of postoperative tumor recurrence is 5% for T1, 10% for T2, and 25% for T3. In case of lymph node involvement, the risk of tumor recurrence increases to 33% for T2 tumor and 66% for T3 tumor. The purpose of preoperative staging of rectal cancer is to predict as accurately as possible the two most important factors for determining the prognosis and risk of recurrence: rectal wall infiltration and regional lymph node metastasis. Accurate preoperative staging of rectal cancer facilitates optimal management, and it helps to determine the need for preoperative neoadjuvant therapy. Those patients whose tumors are confined within the mucosa/submucosa (T1) can be offered local excision as a good alternative to a radical operation. For more advanced rectal lesions, neoadjuvant chemoradiation succeeds in increasing the number of sphincter-preserving operations and improves the local tumor control and survival of these patients.

A number of imaging techniques are now available for staging rectal cancer, including endorectal ultrasonography (EUS), computed tomography (CT), and magnetic resonance imaging (MRI). Endorectal ultrasonography has been the method of choice for the preoperative local tumor staging of rectal cancers. The advantages of EUS are the absence of an irradiation hazard, reducing the discomfort that is associated with the examination, the mobility of the apparatus enabling its use in the outpatient clinic or operating room, and relatively accurate prediction of involvement of the anal sphincter muscle by the tumor. However, there have been some recognized limitations of EUS such as the inability to assess for distant metastases, the inability to accurately stage obstructing lesions due to incomplete luminal passage of the probe, and its high operator-dependency.

INSTRUMENTS AND TECHNIQUE

Endorectal ultrasonography can be performed with either rigid ultrasound probes or with flexible echoendoscopes. While

some endoscopists use an echoendoscope for EUS, most colorectal surgeons and radiologists prefer using the rigid ultrasound probes that are inserted blindly or through a proctoscope. Patient preparation is performed with rectal cleansing 2 h before the procedure by administering two rectal suppositories or a cleansing enema. Sedation is not necessary, and so no specialized monitoring is needed during the procedure. A calm and relaxed ambience helps to put the patient at ease, thereby facilitating the procedure. With the patient in the left lateral decubitus position, a digital rectal examination is performed before the insertion of the ultrasound probe. It allows assessment of the sphincter's tone and palpation of the lesion. If palpable, the lesion should be described in terms of location, distance from the anal verge, and its fixation or mobility.

Rigid ultrasound probes are available as either rotating mechanical scanners or linear array transducers. A transducer emits sounds of varying frequencies; 7.5-, 10-, and 12-MHz radial scanning transducers are usually used. These transducers provide transverse 360° scans in the longitudinal axis of the rectum. Different frequencies change the focal length so that structures can be visualized at different depths. A balloon is placed over the transducer and then properly secured in place. The balloon is filled with 30–60 ml of water that will serve as the acoustic medium for the transmission of the sound waves. All the air is expelled from the system by the repeated insertion and aspiration of water from the balloon. Because any residual air bubbles can result in artifacts, it is important to make sure no air bubbles are present. A condom containing ultrasound gel is placed over the probe. After 50–150 ml of degassed water is instilled in the rectal lumen with an enema syringe, the transducer is then inserted into the anus and advanced into the rectum as deeply as possible. Usually water that is instilled into the rectal lumen during EUS is used to obtain an optimal sonic window. Kim *et al*. (2004b) investigated whether EUS with intrarectal water instillation would improve the depiction and accuracy when staging rectal cancers, and found that the accuracy of EUS in tumor staging was significantly higher after water instillation. This method is thought to be a reliable method of reducing overstaging of rectal cancers because it decreases artifacts that originate from the tumor itself or from feces. In addition, by using the water instillation method, it is possible to easily perform EUS in a distended rectal lumen and to advance the transducer into the upper rectum.

To obtain optimal imaging of the rectal wall layers, certain adjustments are usually necessary, including adjusting the gain of the ultrasound unit and changing the position of the transducer with relation to the rectal wall, thereby bringing the area of interest within the optimal focal distance of the transducer. All the rectal wall layers should be visualized, and once optimal imaging is accomplished, a gradual withdrawal of the probe is initiated. The entire tumor is visualized because there may not be uniformity in the depth of tumor penetration throughout its entire length. Photodocumentation should be obtained and specific notations, such as the distance of the tumor from the anal verge, should be made, or certain anatomic landmarks can be marked on the screen with the keyboard. The size of the tumor or a lymph node can be easily measured using

the calibration device on the ultrasound apparatus. The examination is complete when the entire tumor, rectum, mesorectum, and surrounding structures are thoroughly visualized.

Ideally, the transducer is placed in the middle of the water-filled lumen, perpendicular to the lumen of the bowel and a few centimeters from the tumor. Specific attention is focused on the depth of wall invasion, invasion into the perirectal fat or the adjacent organs (such as the bladder, prostate, seminal vesicles, vagina or anal sphincters), and the presence of perirectal lymph nodes.

IMAGE INTERPRETATION

The normal rectal wall is represented by concentric circles of alternating hyperechoic and hypoechoic bands (Figure 19.1). The rectal wall is divided into five or seven sonographic layers as a result of their differences in acoustic impedance. The first inner hyperechoic layer represents the interface between the ultrasound probe and the mucosa. The second inner hypoechoic layer represents the mucosa and muscularis mucosa. The third middle hyperechoic layer represents the submucosa. The fourth outer hypoechoic layer represents the muscularis propria. The fifth outermost hyperechoic layer represents the interface between the muscularis propria and the perirectal fat. Occasionally, instead of five distinct sonographic layers, it is possible to distinguish seven discrete lines in the rectal wall. Saitoh *et al*. (1986) described the additional hyperechoic line that separates the fourth outer hypoechoic line, and represents the interface between the circular and longitudinal layers of the muscularis propria.

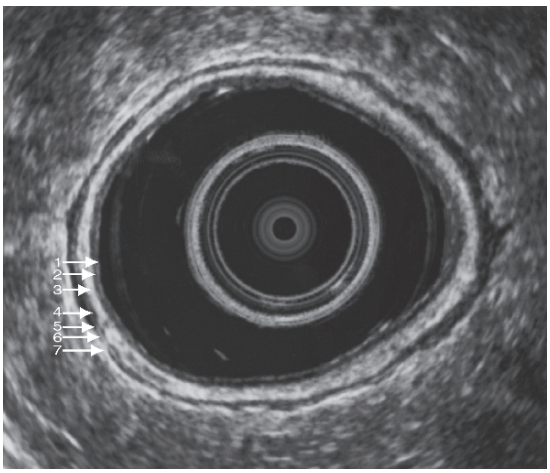

FIGURE 19.1. Endorectal ultrasonography of a normal rectal wall: this is a good example of the seven-layer representation

The ultrasound allows for visualization of the immediate perirectal tissue; thus, a search for enlarged lymph nodes is a routine step in the evaluation of a rectal tumor. Blood vessels may be mistaken for tumor in the lymph nodes, but differentiation is possible if branching or longitudinal extension of the hypoechogenicity is present, and the appearance of this favors a blood vessel. The differentiation between an inflammatory lymph node versus a metastatic node can be difficult at times. However, an enlarged lymph node that is located adjacent or superior to the level of the tumor, has a round appearance with irregular borders and has the same hypoechoic echogenicity as the primary tumor and should be considered to be a metastatic node (Hildebrandt *et al*., 1990). The endorectal ultrasonography also provides an image of the organs adjacent to the rectum. In men, the seminal vesicles are clearly observed and they must be distinguished from lymph nodes. The prostate is also clearly observed, and any tumor

invasion through Denonvillier's fascia can be easily recognized.

TUMOR STAGING WITH ENDORECTAL ULTRASONOGRAPHY

Rectal cancer appears on EUS as a hypoechoic lesion that abruptly interrupts the normal sequence of layers. The endorectal ultrasonography classification of tumor invasion into the rectal wall corresponds to the pathologic T classification of the TNM system. Sonographic local staging of tumor is denoted by the prefix 'u'. The stage uT1 represents mucosal or submucosal tumor seen as a hypoechoic mass with an irregular and thinned middle hyperechoic layer (submucosa) (Figure 19.2). Complete disruption of the submucosa, often with thickening of the muscularis propria, indicates a stage uT2 tumor (Figure 19.3). Stage uT3 is defined by extension through the muscularis propria into the perirectal fat. The thin and hyperechoic outer layer is completely disrupted (Figure 19.4). Stage uT4 represents tumor extension into the adjacent organs or the pelvic sidewall structures.

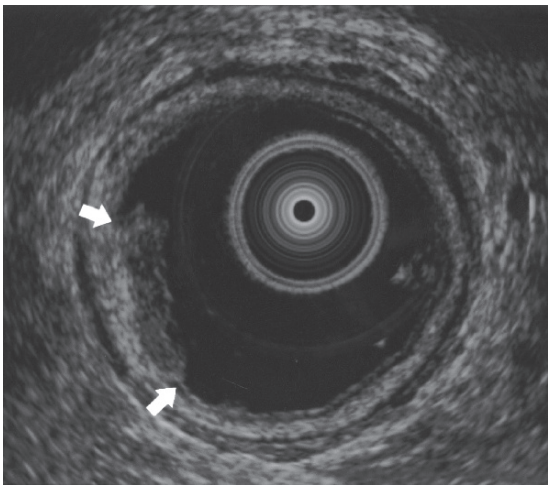

FIGURE 19.2. Endorectal ultrasonographic image of a T1 tumor (arrow). The tumor is confined by the submucosa

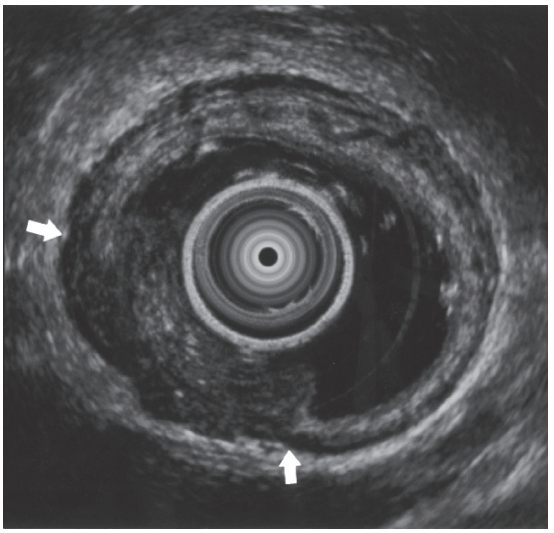

FIGURE 19.3. Endorectal ultrasonographic image of a T2 tumor (arrow). The tumor extends through submucosa into the muscularis propria layer

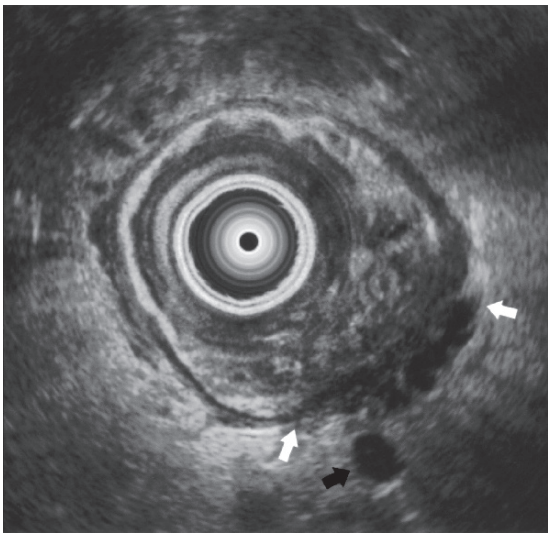

FIGURE 19.4. Endorectal ultrasonographic image of a T3 tumor. The tumor extends through the muscularis propria into the perirectal fat (white arrow). Enlarged lymph node (black arrow)

LYMPH NODE STAGING WITH ENDORECTAL ULTRASONOGRAPHY

Assessment of nodal metastases is difficult as most small lymph nodes are not easily observed while performing EUS. The ultrasonographic criteria for involved nodes include a hypoechoic appearance, a size > 3 mm, a round rather than oval shape (Kim and Wong, 2000), irregular margins (Massari *et al*., 1998), and a lack of an echoic central area (Kumar and Scholefield, 2000). The sonographic staging of lymph node metastasis is interpreted as follows: (1) uN0, no definable lymph nodes by ultrasound and (2) uN1, ultrasonographically apparent lymph nodes.

ACCURACY FOR STAGING RECTAL CANCER

The accuracy rates of EUS for evaluating the depth of tumor invasion have ranged from 63% to 96% (Table 19.1). There is a wide range for the accuracy of EUS. Staging inaccuracies can occur due to overestimation or underestimation of tumor depth, misinterpretation of lymph node involvement, and the operator's inexperience. Endorectal ultrasonography tends to overstage cancers because high-resolution ultrasound can detect, but not separate, inflammation adjacent to the malignancy from the tumor itself. The most common error is overstaging T2 lesions as T3. This error reflects the difficulty of identifying tumor invasion beyond the muscularis propria. The presence of tumor-induced thickening, inflammatory change, desmoplastic reaction or hypervascularity tend to result in the tumors being overstaged in terms of the tumor depth because the echogenecity of tumors is similar to that of both the muscularis propria and the inflammatory infiltrate. Overstaging may also be caused by preoperative biopsy, previous local excision or preoperative radiotherapy, which all cause hemorrhage or local inflammation with obliteration of the sonographic layers of the rectal wall. All of these errors appear as hypoechoic areas and they can easily be confused with cancer. After performing local excision of rectal cancer, postoperative scarring, tissue distortion, displacement of adjacent organs, and the sequelae of local sepsis can all complicate interpreting the postoperative images. Tumor irradiation causes the most significant peritumoral inflammation. After radiation therapy, the rectal wall becomes inflamed, thickened and more hypoechoic, making it difficult to distinguish the different wall layers.

Understaging of tumors may be encountered, but this is less common.

TABLE 19.1. Accuracy of EUS for staging rectal cancer.

Reference	Year	No.	T stage	N stage
Saitoh *et al*.	1986	88	90%	75%
Hildebrandt *et al*.	1986	76	88%	–
Beynon *et al*.	1989	100	93%	83%
Rifkin *et al*.	1989	102	72%	81%
Orrom *et al*.	1990	77	75%	82%
Glaser *et al*.	1990	86	88%	80%
Herzog *et al*.	1993	111	89%	80%
Sailer *et al*.	1997	160	77%	83%
Massari *et al*.	1998	75	90%	76%
Kim *et al*.	1999	89	81%	63%
Akasu *et al*.	2000	154	96%	72%
Marusch *et al*.	2002	422	63%	–
Garcia-Aguilar *et al*.	2002	545	69%	64%
Mackay *et al*.	2003	433	89%	66%
Bali *et al*.	2004	33	79%	59%
Average accuracy			83%	74%

Understaging is more likely to affect the patient outcome than is overstaging. The consequences of understaging are inadequate treatment of a tumor, which may then require a second procedure. Understaging may be caused by a failure to detect microscopic cancer infiltration owing to the limits of resolution of the equipment. In addition, understaging commonly occurs in the case of stenotic lesions, in which the entire tumor may not have been examined.

Another variable that influences the accuracy of tumor staging is operator experience. Orrom *et al.* (1990) reported an increase in accuracy from 59.3% to 95% during a period of 3 years, during which 30 cancers per year were examined. Marusch *et al.* (2002) showed considerably lower accuracy rates in a prospective multicenter study involving 75 hospitals. The authors stressed the need for highly trained operators with large caseloads, and they concluded that centralization of EUS service is mandatory if a high level of quality is to be achieved with employing this method. Using the data of a large single institution, Mackay *et al.* (2003) suggested a learning curve of up to 50 cases for accurately detecting tumor penetration and more than 75 cases for accurately assessing the node status.

Because the ultrasound probe will not pass beyond a stenotic tumor, such lesions cannot be adequately evaluated with EUS. Hawes (1993) reported that stenotic lesions make up > 17% of rectal cancers. Using a hard plastic cap rather than a balloon may be helpful for examining stenotic lesions. In such a situation in women, using a water enema and a transvaginal ultrasound examination is a valuable technique to define the local extension of severely stenotic rectal cancers. For a more proximally located cancer, a specially designed rigid sigmoidoscope inserted under direct vision facilitates the examination by passing the ultrasound probe through the sigmoidoscope beyond the cancer. Inadequate bowel preparation can lead to reverberation artifacts; these are caused by feces obscuring the tumor margin. This can be overcome by administering a phosphate enema to cleanse the rectum prior to the test.

The overall accuracy rates for assessing nodal metastases range from 63% to 83%, with an average accuracy of 74% (Table 19.1). Determining lymph node involvement is less precise than that of tumor staging. The lower nodal staging accuracy is attributed to the observation that up to 50% of malignant nodes are < 5 mm in diameter and the rate of EUS detecting these nodes may be as low as 20% (Spinelli *et al.*, 1999). The overstaging is primarily caused by the presence of reactive swollen lymph nodes. The reasons for understaging are: (1) the difficulty in detecting very small involved nodes, (2) the lateral pelvic lymph nodes, like the obturator nodes, are located so far from the rectum that they cannot be effectively imaged with the currently available probes, and (3) the inadequacy of the criteria for the involved node (Kim *et al.*, 2001).

Beynon (1989) used shape and hypoechogenicity as markers of nodal metastasis and reported an accuracy of 83%. Based on the presented data, no lymph node > 8 mm was noted to be falsely-positive or negative. On the other hand, Hildebrandt *et al.* (1986) used the degree of echogenecity alone. There is also a discrepancy of the diameter used as a criterion for nodal metastases. However, the size of the lymph node is of little value for differentiating malignant from reactive

lymphadenopathy. Katsura *et al*. (1992) reported that 18% of lymph nodes < 5 mm harbor metastases and these small lymph nodes are not readily identified sonographically. Thus, recent studies suggest that multiple criteria, including size, shape, and the outer borderlines of a lymph node should be used to improve accuracy. Lymph nodes located in the periphery of the mesorectum may also remain undetected if they exceed the depth of penetration of the transducer.

ACCURACY OF ENDORECTAL ULTRASONOGRAPHY COMPARED WITH COMPUTED TOMOGRAPHY AND MAGNETIC RESONANCE IMAGING

Many studies have attempted to evaluate whether the EUS staging is better than that done *via* CT or MRI. Computed tomography scanning for staging rectal cancer can evaluate local wall involvement, the perirectal lymph nodes, and distant metastases. Several comparative studies have shown EUS to be superior to CT in terms of both T and N staging (Table 19.2). For the T staging of rectal cancer, the accuracy of EUS has been found to range from 72% to 91%, whereas CT has been found to have accuracies ranging from 53% to 94%. For the N staging of these tumors, the accuracy of EUS has been found to range from 61% to 81%, whereas CT has been found to have accuracies ranging from 56% to 72%. Kwok *et al*. (2000) reported in a meta-analysis of 78 studies, including 4,897 patients with rectal cancer, that CT showed an accuracy of 73% for T staging and 66% for N staging, respectively. Computed tomography has low spatial resolution and so it cannot define the layers of the rectal wall, resulting in variable accuracy rates according to the T stage. For determining of the N staging, CT evaluation is usually based on size alone and therefore, is not reliable. With the advent of multi-detector CT, the spatial resolution has improved considerably due to the thinner collimation and the improved multiplanar images. Multi-planar images can be potentially useful for staging as they can be aligned parallel or perpendicular to the axis of the tumor. However, there are only a few studies that have addressed the current role for multi-detector CT and the inherent low contrast resolution still remains as its limitation. Multiple prospective studies should be done to determine the role of multi-detector CT for staging.

TABLE 19.2. Accuracy of EUS versus CT.

Reference	Year	No.	EUS T stage	CT T stage	EUS N stage	CT N stage
Beynon *et al*.	1986	44	91%	82%	–	–
Holdsworth *et al*.	1988	36	86%	94%	61%	70%
Waizer *et al*.	1989	68	76%	66%	–	–
Rifkin *et al*.	1989	81	72%	53%	81%	72%
Herzog *et al*.	1993	87	91%	75%	–	–
Osti *et al*.	1997	63	83%	74%	66%	57%
Kim *et al*.	1999	89	81%	65%	63%	56%
Average accuracy			83%	73%	68%	64%

Several previous studies have compared the accuracy of EUS with that of MRI (Table 19.3). Magnetic resonance imaging has a similar accuracy to EUS for staging rectal cancer. For the T staging, the accuracy of EUS has been found to range from 64% to 88%, whereas MRI has been found to have accuracies ranging from 64% to 85%. The development of endorectal coils has improved the accuracy of MRI, and further developments in phased-array coils have led to better spatial resolution and accuracy for predicting the tumor stage. Although the overall T staging accuracies via MRI are similar to those of EUS, MRI has higher accuracies when assessing T3 and T4 tumors as compared to assessing T1 and T2 tumors. Mathur *et al*. (2003) showed accuracies of 43% for T1 and T2 tumors, and 76% for T3 tumors. Endorectal ultrasonography can accurately stage the depth of tumor invasion particularly for T1 and T2 rectal cancers, whereas MRI seems superior for more locally advanced disease (T3 and T4). For the N staging, the accuracy of EUS has been found to range from 54% to 80%, whereas MRI has been found to have accuracies ranging from 60% to 81%. While EUS applies the criteria of a lack of ovoid morphology and a central echogenic nidus, high-resolution MRI with its ability to depict the inherent contrast between fat and lesions, accurately predicts nodal involvement when the morphological features, such as a speculated or indistinct border and a mottled heterogeneous appearance, are used rather than using the nodal size alone (Kim *et al*., 2004a).

THREE-DIMENSIONAL ENDORECTAL ULTRASONOGRAPHY

Three-dimensional (3D) EUS is a new technique that is still undergoing development. Three-dimensional EUS enables a multi-planar display that consists of coronal and sagittal scans in addition to a transverse scan. The minimal slice thickness obtained with 3D EUS provides enhanced resolution with a smaller voxel size than the other imaging tools can achieve. Assessment of additional scan planes and volume reconstructions facilitate the understanding of the three-dimensional anatomy and it improves evaluating the depth of tumor invasion. Hunerbein and Schlag (1997) reported that 3D EUS allows the visualization of obstructing tumors with using reconstructed planes in the front of the transducer, and it also enables precise transrectal biopsy of suspicious

TABLE 19.3. Accuracy of EUS versus MRI.

Reference	Year	No.	EUS T stage	MRI T stage	EUS N stage	MRI N stage
Thaler *et al*.	1994	37	88%	82%	80%	60%
Starck *et al*.	1995	35	88%	66%	71%	72%
Kim *et al*.	1999	89	81%	81%	63%	63%
Gualdi *et al*.	2000	26	77%	85%	72%	81%
Maldjian *et al*.	2000	14	71%	71%	54%	77%
Fuchsjager *et al*.	2003	39	64%	64%	70%	62%
Bianchi *et al*.	2005	49	70%	71%	63%	76%
Average accuracy			77%	74%	68%	70%

pararectal lesions. Giovannini *et al*. (2006) reported that the mesorectal margins are defined better with 3D EUS than with using conventional EUS. Kim *et al*. (2002) evaluated 3D EUS and found that 3D EUS identified more mesorectal lymph nodes than did conventional EUS. Kim *et al*. (2006) also reported that 3D EUS showed greater accuracy than conventional EUS or CT for rectal cancer staging (78%, 69%, and 57%, respectively) and lymph node metastases (65%, 56%, and 53%, respectively). It seems likely that 3D EUS is capable of improving the staging of rectal cancer and this technique will become a valuable adjunct to conventional EUS.

In conclusion, EUS is a valuable diagnostic method that can contribute to the preoperative staging for rectal cancer and can be a guide to determine the appropriate treatment for either early or advanced disease. The newer modalities such as 3D EUS may further improve the accuracy of this modality. The combination of EUS with other diagnostic methods may greatly aid physicians to more accurately predict the local stage of rectal cancer.

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20
Rectal Cancer: Spectral Imaging and Immunohistochemistry of Thymidylate Synthase

Gary K. Atkin and George D. Wilson

INTRODUCTION

Thymidylate Synthase

In order to synthesize DNA, proliferating cells require a continuous supply of deoxyribonucleotides. Thymidylate synthesis involves the reductive methylation of deoxyuridine-5'-monophosphate (dUMP) to deoxythymidine-5'-monophosphate (dTMP) (Radparvar *et al*., 1988). The enzyme that catalyzes this conversion is thymidylate synthase (TS). This is a cytosolic enzyme, existing as a 77 kDa dimer. It catalyzes the reaction that provides the only *de novo* source of the thymidine nucleotide needed for DNA synthesis. It is, therefore, the rate-limiting step in thymidine biosynthesis (Aschele *et al*., 2002), and has been investigated as a target for a number of chemotherapy agents. This enzyme has been shown to be the main site of action of 5-fluourouracil (5-FU) (van der Wilt and Peters, 1994), which has been the major chemotherapy agent used in patients with colorectal cancer (CRC) for many years. 5-Fluourouracil is converted within cells to its active metabolite flourodeoxyuridine monophosphate (FdUMP) which then competes with the natural substrate for TS (dUMP) forming a stable ternary complex (Santi *et al*., 1974).

As TS is intimately related to the action of 5-FU, it has been extensively studied as a prognostic marker and a marker of response to 5-FU based chemotherapy in rectal cancer (Johnston *et al*., 1994; Edler *et al*., 2000; Okonkwo *et al*., 2001). Generally, low TS levels predict a better outcome to 5-FU based chemotherapy, whilst high TS expression is associated with a poor prognosis. A recent meta-analysis included 20 studies in CRC patients in which overall survival and/or progression-free survival was stratified by TS expression status (Popat *et al*., 2004). Of these studies, 13 investigated outcome in a total of 887 cases with advanced CRC, whilst 7 investigated outcome in 2,610 patients with localized CRC. The methods used to determine TS expression and assign expression status were immunohistochemistry (IHC), reverse transcriptase polymerase chain reaction (RT-PCR), and enzyme assay. In all but two studies, TS expression was assessed by one method only. In these two studies, although two methods were applied, only one was used to assign TS status for subsequent survival analysis (IHC or RT-PCR, respectively). As with many prognostic marker studies, both sample size and cut-off values varied

greatly, with small sample size in particular being a feature of the advanced CRC studies. The combined hazard ratio (HR) estimate for overall survival was 1.74 (95% CI, 1.34–2.26) and 1.35 (95% CI, 1.07–1.80) in the advanced and adjuvant settings, respectively. Thus, tumors expressing high levels of TS appeared to have a poorer overall survival compared with tumors expressing low levels. The recommendation from this meta-analysis was that additional studies with consistent methodology were still required to define the precise prognostic value of TS.

The mechanism of TS overexpression can occur as a result of deregulation of cell cycle control, resulting in activation of the transcription factor and oncogene E2F-1. In this scenario, TS may act as a downstream effector of E2F-1 (Banerjee *et al*., 2002). In addition, the TS gene promoter enhancer region contains two different polymorphisms that can influence TS mRNA transcriptional and translational efficiency: a polymorphic tandem repeat sequence (2R or 3R repeats) and a single nucleotide polymorphism (SNP), G > C, within the second repeat of the 3R alleles. Results suggest that promoter polymorphisms may be important in determining TS mRNA expression levels and are associated with sensitivity to 5-FU-based chemotherapy (Curtin *et al*., 2007).

Several techniques have been used to investigate the predictive ability of TS, including biochemical assays, RT-PCR and IHC. The optimal technique has yet to be determined, but IHC is probably the most practical and widely available for human tumor samples. It is inexpensive and is performed on paraffin-embedded samples, the main medium of archival storage of human tumors. Thymidylate synthase protein expression has been shown to correlate approximately with TS gene levels (Johnston *et al*., 1995), and the regulation of TS expression occurs mainly at the level of gene translation (Jenh *et al*., 1985); hence, TS protein measured by immunohistochemistry is a direct and accurate assessment of TS gene expression.

Immunohistochemical Quantification of Tissue Sections

Immunohistochemical quantification is becoming increasingly important to investigate the correlation between pathological data and clinical outcomes. Therefore, accurate interpretation of immunostaining is vital in order to dictate therapeutic strategy. Traditional methods of immunostain quantification have included visual estimates of stain intensity and visual scoring techniques, such as counting positive vessels for microvessel density estimates or scoring the percentage of positively and negatively stained cells. These traditional methods are subjective with considerable interobserver variability (Levenson and Hoyt, 2000). In the case of TS, both polyclonal and monoclonal antibodies have been used for primary detection, which introduce another potential source of variability between studies; standardization of future studies by the use of a single monoclonal antibody is important.

Figure 20.1 shows a collage of images exemplifying the variability of TS expression in CRC in terms of stain intensity, positivity, and distribution. As with most markers associated with proliferation, it is rare to find a tumor with no expression of TS. There is a high degree of variability in stain intensity between different tumors, from weak (Figure 20.1a) through moderate (Figure 20.1b)

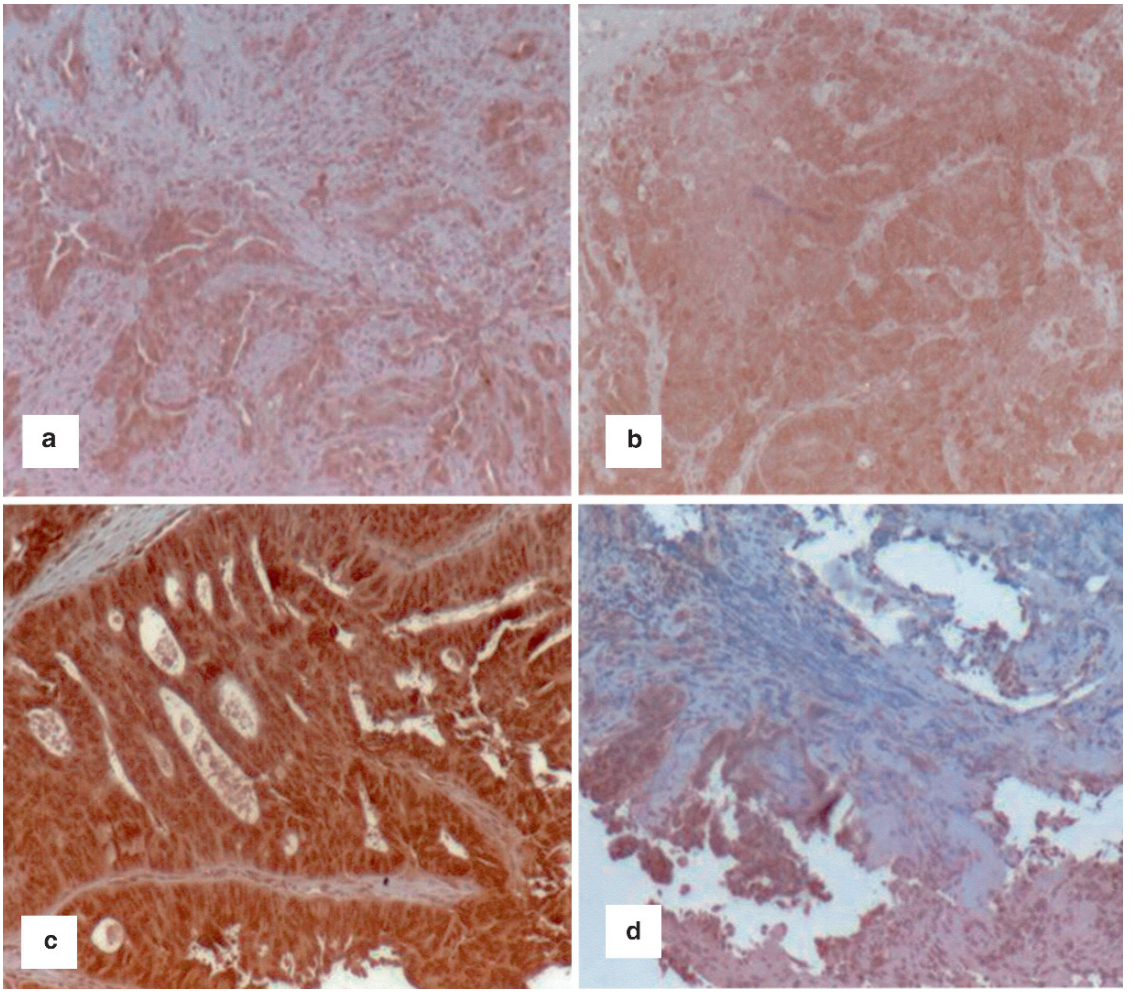

FIGURE 20.1. Examples of TS staining intensities and positivity scores for colorectal cancer (see text for description)

to strong (Figure 20.1c). Although batch-to-batch variation in antibodies and reagents and day-to-day variation in staining can contribute to differences in staining intensity, there is a consistent and reproducible variation in stain intensity and thus, levels of TS protein expression. In conjunction with the variation in stain intensity, there is also considerable variation in the percentage of tumor cells positive for TS. In our study (Atkin *et al*., 2005), 9% of images contained < 20% positive cells (Figure 20.1d), and 24% had between 20% and 50% positive cells (Figure 20.1a). Two thirds of patients had > 50% positive cell staining, with one third having > 75% (Figure 20.1c). This demonstrates the wide variation in TS expression found in colorectal cancer.

Various authors have used different criteria to grade tumor expression. In some studies, tumor expression has been graded

from 0 (no staining) to 4 (maximum staining). Other studies take into account the percentage and nature of staining (focal or diffuse, nuclear or cytoplasmic). In common with most proteins, extremes of expression appear easy to distinguish, but difficulties arise in tumors with intermediate expression grades, and these may be more difficult to correlate with outcome. Clearly, there is not yet a gold standard for assay of TS expression, and comparative assays on tumors are needed to settle this issue. One idea is the establishment of a central referral laboratory for the measurement of TS at both the mRNA and protein levels, so that variability between the assay methods can be avoided. Whether cDNA microarrays and tissue microarrays will be better than quantitative RT-PCR and laser microdissection and IHC, respectively, will have to await method validation studies and acceptance by other laboratories engaged in such studies.

Consequently, other quantification methods have recently been developed. Computer assisted image analysis is a technique aimed at improving automation and reproducibility. It utilizes a microscope, camera, and a computer to produce a digital image representing the microscopic field. The image is divided into picture elements (pixels) and for each pixel the amount and color of light captured by the camera is calculated. This information can then be displayed on a grey or color scale. This allows separation (segmentation) of cells or regions, which can be done automatically by setting a threshold value. The technique separates cellular staining from background staining by setting certain parameters to define a cell within the microscopic field. This is more difficult when several cells overlap within a region, but quantification is still possible by manual segmentation of cells or by the use of edge-finding algorithms.

Computer assisted image analysis has been applied to the fields of analyte concentration determination and morphometric analysis (Aziz and Barathur, 1994). The concentration of analyte is calculated by determining the absorbance of light by the histological section. The technique has been used in breast cancer to derive data on nuclear antigens, such as estrogen receptor (ER) status, and cytoplasmic antigens, such as HER-2/*neu* (Aziz and Barathur, 1994). The latter is more complex and requires algorithms to detect small circular regions of staining surrounding a nucleus. It may be facilitated by manually selecting the regions of interest, but this decreases automation. The introduction of image analysis for ER status determination has allowed much smaller samples, as well as paraffin-embedded archival sections, to be assessed compared with the previous technique of enzyme immunoassay.

Morphometric analysis measures histocytological features by quantifying patterns, shapes, and textures of biological materials. For example, specific algorithms have been used to measure nuclear pleomorphism in prostate cancer by calculating the variance of nuclear shape from a perfect circle (Partin *et al*., 1989). Similarly, stromal-to-epithelial ratio and percentage tumor involvement within a section have been determined (Wied *et al*., 1989).

Thymidylate synthase expression has been studied using computer-assisted image analysis in a small series of patients (Bendardaf *et al*., 2005). In this analysis four low power images were captured from each slide covering most of the tumor area, and using software from Imaging Research Inc. (now Interfocus Imaging Ltd.), the diaminobenzidine chromogen associated

with positively stained cells was measured. Details of the methodology are scant, but it is likely that the optical density of the stained cells was measured, as well as the total area stained on a pixel-by-pixel basis. Using this method, it was possible to distinguish between the presence of many cells expressing low amounts of TS and a few cells expressing high amounts, such that the percentage of TS expression reflected total TS expression in the tumor. The authors speculated that this approach may be more relevant biologically. However, although their data showed a statistically significant association between TS expression and response to 5-FU, folinic acid, and irinotecan, the data were similar to other studies in which visual scoring was employed.

As with visual estimation techniques, computer assisted image analysis is also prone to the limitations of nonspecific antibody binding and the variations in staining protocols that reduce reproducibility. The problem of interlaboratory variation also persists and has driven the search for standardisation of techniques between centers, possibly by distributing standard control sections used to calibrate individual instruments. Image analysis is inferior to interpretation by an experienced pathologist, but it allows automation, and if the methods are standardized it ensures intra- and interobserver subjectivity and variability are minimized. It is important to validate any image analysis system by comparison with the manual assessment by a histopathologist (Aziz and Barathur, 1994).

Spectral Imaging

Spectroscopy measures the characteristics of light impinging on an object. It is used to determine the physical or chemical properties of the illuminated object, and so can provide information on the constituents of a tissue section containing an immunostain of a particular color. Optical imaging applied to spectroscopy allows this information to be displayed visually. Human color vision is a form of imaging spectroscopy that determines the proportion and intensity of wavelengths present in the field of vision. It is limited, however, by the need to separate the light content of an image into only three broad wavelength bands, representing red, green, and blue (RGB) colors. This is similar to the principle of a conventional RGB camera. Details of the actual color spectrum are therefore lost once it is divided into these three bands. Spectral imaging allows light to be separated into an arbitrarily large number of wavelength bands. It can also measure light in the ultraviolet (UV) and infrared (IR) regions of the visible spectrum, both of which are invisible to the naked eye (Farkas and Becker, 2001). It is able to generate qualitative and quantitative images of the object under investigation, as well as delivering automation to the field of microscopy (Barber *et al*., 2003). Spectral imaging allows a high-resolution spectrum of light intensity as a function of wavelength to be produced for each pixel of the image (Figure 20.2) (Farkas and Becker, 2001). This individual spectrum with its characteristic pattern is termed the *spectral signature* for that pixel. When all pixels are analysed, the image can be represented as a spectral cube with x, y, and wavelength representing the three axes. This provides spatial information within the image of the intensity of light at any particular wavelength.

The application of spectral imaging to the quantification of immunostaining is based on this ability to measure the intensity of light for each wavelength at each image

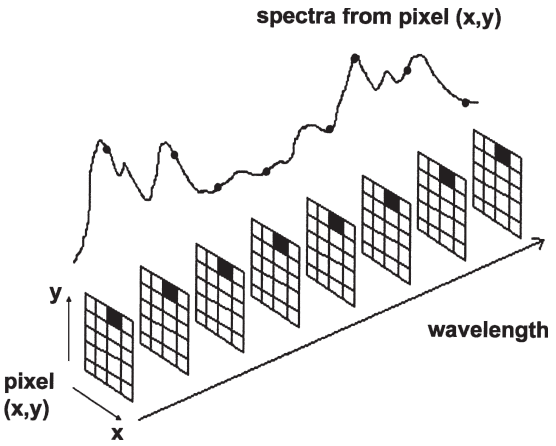

FIGURE 20.2. Principles of spectral imaging. A high-resolution spectrum of light intensity against wavelength is produced for each pixel of the captured image.

pixel. For the dyes used in immunohistochemistry, each has a specific shape to their absorption spectrum (Barber *et al*., 2003). Once these spectra are known, they can be modelled on the spectrum produced at each pixel by the spectral imager, and so the contribution of each dye to the spectrum at that pixel can be determined. Hence, the amount of dye present within the section is quantitatively assessed (Farkas and Becker, 2001). Most dyes have very broad absorption spectra, which limits the utility of a conventional RGB camera. The spectral imager provides information on the intensity of each wavelength studied, and this improved detail allows multiple dyes within a section, which may have similar but individual spectra, to be separated and individually measured.

Changes in cellular constituents in inflammatory or neoplastic cells affect the distribution, intensity, and color of routine histological stains. This principle may by utilized by spectral imaging to provide qualitative data on biological tissue sections. For example, the Papanicolaou stain uses haematoxylin, orange G, eosin Y, and light green stains, and differences in cellular atypia can be detected by subtle differences in the color of the nuclei (Farkas *et al*., 1998). Similarly, the characteristic spectral signatures of cells within conventionally stained sections have been used to differentiate pre-neoplastic melanoma cells from normal cells of the same lineage using spectral imaging (Levenson and Hoyt, 2000). This provides information usually only available with special immunostains. Spectral imaging has also been used in melanoma to improve detection of early stage disease and determine gene expression profiles (Farkas and Becker, 2001).

Multiple dyes staining different markers within the same section can be segmented on the basis of their spectral signature. They can then be analyzed qualitatively (for example, the distribution of carbonic anhydrase-9 [CA-9] staining around blood vessels) or quantitatively by determining the amount of each dye throughout the section. This has been used in breast cancer to determine the ER status (Rothmann *et al*., 2000). We have previously investigated spectral imaging as a method of quantification of TS staining in rectal cancer (Atkin *et al*., 2005). The system was validated by comparison with the technique of manual visual grading. The methodology of the spectral imaging system used is given below, along with the protocol for TS staining in rectal cancer.

METHODOLOGY

Immunohistochemistry of Thymidylate Synthase in Rectal Cancer

Human tumor samples were fixed in 10% neutral buffered formalin solution for 24 h. The specimens were dehydrated in graded alcohols, washed with xylene and

embedded in paraffin. Then, 4 μm sections were dewaxed in xylene for 5 min and rehydrated through graded alcohol (100%, 90%, and 70%) to water. Heat mediated antigen retrieval was performed by placing the sections in 250 ml of 10 mM citric acid (pH 6) and boiling for 4 × 4 min in an 800 W microwave oven. A constant volume of 250 ml was maintained by replacing evaporated fluid with distilled water between heating cycles. The sections were left to stand in citric acid for 10 min before washing in water for a further 5 min.

Sections were then transferred to the DAKO Autostaining machine (DAKO, UK) containing peroxidase block (DAKO, #S2023), the detection reagents (Chemate HRP, DAKO, #K5001), and anti-human primary antibody (gift of Simon Joel, London, UK) diluted 1:300 in antibody diluent. The Autostainer programme included 5 min in peroxidase block, 1 h incubation in primary antibody, 30 min incubation in ChemMate secondary and tertiary reagents and 5 min in diaminobenzidine (DAB) substrate. When the program was complete, stained slides were removed from the machine and counterstained in Gills hematoxylin (Surgipath Europe Ltd, #01500E) for 5 s. Slides were washed in tap water, dehydrated in graded alcohols (70%, 90%, and 100%), cleared in xylene and mounted in DPX (Surgipath Europe Ltd, #08600E). Each staining run incorporated a control slide (breast or colorectal carcinoma) that had previously demonstrated positive for TS. A negative control was also incorporated and involved the substitution of the primary antibody for an isotypic control antibody at the same protein concentration.

Quantification of Thymidylate Synthase Expression Using Spectral Imaging

Immunohistochemical staining of marker protein expression was quantified using a spectral imager developed and constructed in our Institute (Barber *et al*., 2003). The spectrally selective element was placed between the camera and the microscope output port using standard C-mount couplers and was based on a linearly variable dielectric bandpass filter together with novel drive hardware and acquisition software. The element had a resolution of 15 nm and covered the 400–700 nm band with a transmission of > 40%. The spectrally resolved device was used with an upright microscope (Optiphot, Nikon, UK), and a range of achromatic objective lenses was used: 1.6x, 10x and 20x.

Images were captured into a personal computer via a frame grabber (type PCI-1409, National Instruments Ltd, UK). Software was written in 'C' programming language under the Lab Windows/CVI™ development environment (National Instruments Ltd, UK) and Windows 2000 operating system (Microsoft Corp, USA). The relationship between absorbance and analyte concentration is described by Beer's law, which states that at any wavelength (λ) light absorbance (A) is proportional to the concentration of the absorbing medium (C) and the thickness of the sample (d):

$$A_{(\lambda)} = E_{(\lambda)} \text{ x } C \text{x } d$$

where E is the wavelength-dependant extinction coefficient. The absorbance is often referred to as optical density (OD), and the relationship between the light transmittance (T) and intensity (I) through the sample and the OD at a given wavelength is described by Lambert's law:

$$OD = \log_{10} T_{(\lambda)} = \log_{10} \left[I_{transmitted(\lambda)} / I_{incident(\lambda)} \right]$$

These equations are combined and known as the Beer–Lambert Law:

$$OD(\lambda) = E_{(\lambda)} \times C \times d$$

The optical density (OD) at a given pixel was determined for each section using the following equation:

$$OD(\lambda) = \frac{-\log_{10} I(\lambda) - I_{black}}{I_{black}(\lambda) - I_{black}}$$

where I_{blank} is the intensity through a blank part of the section (of equivalent optical thickness), and I_{black} was the intensity with no illumination. The concentration (C) of immunostain present within the section was thus determined from the equation $OD(\lambda) = E_{(\lambda)} .C . d$ (where thickness, d, was constant).

The characteristic spectra of the dyes used in the immunohistochemical process (DAB, hematoxylin, and eosin) were generated by analyzing sections stained only with the single dye. These spectra were then used to determine the contribution of each dye to the spectra at each pixel of the sample image by applying a non-negative least squares unmixing algorithm (Lawson and Hanson, 1974). The results of the OD spectral un-mixing represent proportions of the reference spectra, and as such have arbitrary units (a.u.) of OD normalized to the references. Frequency histograms of normalized OD were generated for DAB staining for each section (Figure 20.3), and OD data were presented as grey-scale intensity maps for the whole image.

From the frequency histogram, it was possible to derive the mean normalized OD representing the stain intensity for that image. This allowed objective quantification of the intensity of marker protein

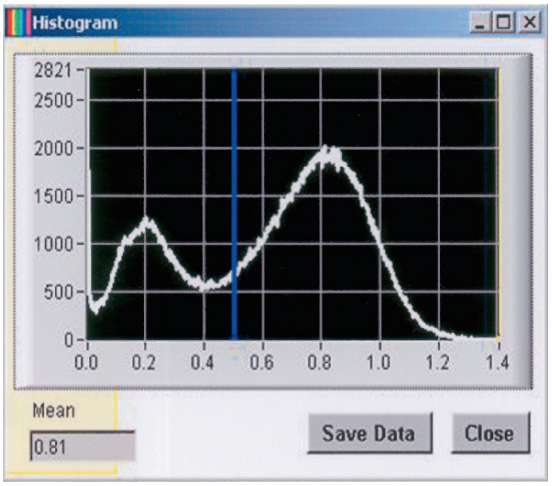

FIGURE 20.3. Spectral imaging produces a histogram of frequency (y-axis) against normalized optical density (OD) (x-axis), from which the mean stain intensity and area are calculated. An arbitrary threshold (blue line) is used to segment the image.

expression. The area under the frequency histogram curve was also calculated and represented the number of pixels, and therefore the area of the captured image, demonstrating marker protein expression.

The grey-scale intensity maps allowed spatial correlation of stain intensity with the histocytological architecture. It was evident that DAB uptake is seen in stromal tissue even though this is not visible on the hematoxylin and eosin-stained section. This is because all areas of the section take up DAB, hematoxylin, and eosin to a greater or lesser extent. The improved detection capabilities of the spectral imager allowed visualization of this previously undetectable dye uptake.

Thymidylate synthase is preferentially expressed in colorectal cancer compared with normal colonic mucosa (Paradiso *et al*., 2000), but DAB is taken up by all areas, so the frequency histogram revealed two peaks (Figure 20.3). The larger peak

at higher OD represented the immunostain within tumor tissue, whereas the lower peak corresponded to the stromal staining. An arbitrary threshold was chosen to segment the image and minimize involvement of this background staining in the final analysis. This allowed selective measurement of the tumor protein expression. The same threshold was applied to all images.

DISCUSSION

Spectral imaging is a useful and attractive form of image analysis, and appears suitable for both bright field and fluorescence microscopy. As with other forms of microscopy, it has the advantage over methods of cellular quantification such as flow cytometry, in that the features to be assessed can be correlated with the tissue microarchitecture. It also has the advantage over standard color imaging in that its ability to measure a large number of wavelengths at each pixel enables the image to be spectrally resolved. This allows histological dyes with characteristic spectra to be separated, thereby permitting the simultaneous measurement of multiple analytes present within a section (Farkas *et al*., 1998).

As the biology of cancer and the mechanisms of anti-tumoral agents are further elucidated, there will be a greater need for accurate quantification of biological constituents, in order to predict tumor behaviour and permit targeted therapy based on the results of tissue marker quantification. Therefore, there will be a greater role for objective and automatic scoring systems in the future. However, there will always be a role for the manual grading of protein expression, as these techniques are still some way ahead of image analysis in terms of assessing qualitative parameters, such as the association of protein expression with physical features such as the epithelial basement membrane (Mighell *et al*., 1998).

There are inherent limitations to spectral imaging, such as the inclusion of background staining in the quantification of marker expression. This is reduced by the application of a threshold to the normalized OD data, excluding background staining on the grounds of its lower stain intensity. However, this requires greater expression of the protein marker in tumor tissue compared with stroma. For standardization, when sections are being compared against each other it is important to apply the same threshold to all data. This will inevitably exclude weakly stained tumor areas for some sections. Spectral imaging is also prone to errors introduced during immunohistochemistry. Variations in staining occur between different centers, and between runs on subsequent days in the same laboratory despite similar reaction components (Seidal *et al*., 2001). It is vital, therefore, that all sections to be compared are stained together. Excessive staining around the periphery of a section (edge artefact), may erroneously increase spectral stain intensity. This suggests a need for accurate image segmentation, so that user-defined regions can be analyzed independently, or, as with tumor-specific antigens, stromal staining can be excluded on the basis of its lower intensity. However, this is difficult as manual selection of regions would reduce automation, whereas application of thresholds to minimize nonspecific staining would inevitably exclude weakly stained tumor areas.

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B. Treatment

21
Cancer of the Rectum: Abdominoperineal and Sphincter-Saving Resections

Federico Bozzetti

INTRODUCTION

P. Kraske (1875) is generally credited with having performed the first sphincter-saving excision (SSR) of the rectum in 1875, although this operation became unpopular due to the number of complications.

Ernest Miles first described the abdominoperineal resection (APER) in 1908 and for almost a century this operation which implies a total proctectomy and a permanent colostomy had been the gold standard for all malignant tumors of the lower and middle third. The Miles' operation was highly morbid and was accompanied by an operative mortality of 36% in his initial series of 61 patients.

The rationale of the APER mainly relied on biological and technical considerations: firstly, Miles regarded that involvement distal to the level of the tumor was a main route of tumoral spread, and for a long time it was accepted that a distal clearance of several centimetres from the tumor was necessary to achieve a healthy distal margin of transection. Secondly, a low colorectal anastomosis represented before the advent of the mechanical staplers a high risk procedure, which often required a protective stoma and a subsequent operation for its closure, although in many cases temporary stomas become definitive. These two factors often dictated the choice of an APER for all rectal tumors which were appreciated through the digital rectal examination.

These concepts, however, were challenged in the last decades of the past century as a result of better understanding of disease spread and of the anatomopathological distribution of the regional lymphadenopathies and the technologic progress in surgical instrumentation. Moreover, the introduction of total mesorectal excision (TME) and its ability to decrease local recurrence without compromising either oncologic radicality or continence shifted the focus of the surgical interest on the mesorectum and on its adequate dissection rather than on the proper distal clearance of the viscus which could guarantee tumor-free transection margins.

The majority of surgeons now accept that if it is possible to transect the rectum 1 cm distally to the tumor margin, without jeopardizing the sphincter, then a sphincter-saving resection (SSR) – (usually a low or a ultra-low anterior resection of the rectum) – with TME should be performed.

On the other hand, if tumor is fixed to the anal sphincter or is less than 1 cm above the anal sphincter, an APER should be performed.

However, some reports (Heald *et al*., 1997) suggested that APER was associated with local recurrence and inferior oncologic outcomes and postulated that lack of precise definable planes or perineal dissection could explain the higher incidence of local recurrence through the shedding of cancer cells on large areas of raw surfaces by allowing implants or leaving behind tumor-laden soft tissue residues. Presently, it is important to know whether this report dating back 10 years has been confirmed and, if so, whether the worse outcome is dependent on the type of the surgical procedure or on the fact that APER and SSR are operations which are performed under conditions that *per se* are different from the prognostic point of view.

Precise definitions of SSRs for distal rectal tumors are lacking. Following the indications of Tytherleigh and Mortensen (2003) the anal canal is ~ 4 cm long in males and 2.5–3 cm in females, and the rectum is between 12 and 15 cm in length. The junction of the rectum and anal canal occurs at the pelvic floor, that is at level where the puborectalis part of the levator ani clasps the bowel and angles it forwards. The low rectum is generally regarded as 0–5 cm from the anal verge; the mid-rectum, 6–10 cm from the anal verge and the upper rectum, 11 or 12 cm from the anal verge in the left lateral Sims' position on rigid proctoscopy. A low rectal tumor may then be defined as one lying < 5 cm from the anal verge or < 1 cm from the anorectal junction (or ring).

A tumor of the middle third of the rectum may be removed by an anterior resection, a tumor lying in the lower third of the rectum (which does not extend into the distal 1 cm of the rectum) may be removed by a low anterior resection, usually followed by an intrapelvic colorectal anastomosis, whereas the complete excision of the rectum for a low rectal cancer is defined as an ultralow anterior resection. Depending on the site of the anastomosis at just above the junction or beyond the anorectal junction into the anal canal, the anastomosis will be an ultralow extrapelvic colorectal anastomosis or a coloanal anastomosis.

OUTCOME AFTER APER AND SSR

A review of the literature regarding local recurrences after APER and SSR was published in 1996 (Bozzetti *et al*., 1996) and included more than 1,400 patients (from 12 different series) who underwent surgical procedures for cancer of the middle and low rectum. Four studies reported a statistically higher rate of pelvic recurrences after SSR than after APER and no study demonstrated an advantage for local recurrence with SSR. In our institutional analysis of 350 patients with cancer of the low and middle rectum, the multivariate analysis showed that SSR was associated with 2.6 times higher risk of pelvic recurrences (P = 0000.1), but with no excess of 5-year mortality.

The dissociation between pelvic recurrences and overall mortality was apparent also in the National Surgical Adjuvant Breast and Bowel Project study (413 patients) where 4-year survival was similar despite the higher prevalence of pelvic recurrences in SSR patients (Wolmark and Fisher, 1986).

These findings, however, were challenged by the more recent experience of

the surgical treatment of the rectal cancer which also focused on the TME as the cornerstone in the treatment.

Survival and recurrence rate reported in the literature of the last decade are summarized in Tables 21.1 and 21.2.

Survival was better after SSR than after APER in five out of nine studies. Local recurrences were equally distributed in seven studies, were more frequent in one study with SSR and more frequent in two studies with APER. It is noteworthy, however, that in three extensive series the benefit in survival (Park *et al*., 1999) and in local recurrences (Wibe *et al*., 2004; Law and Chu, 2004) with SSR disappeared at the multivariate analysis after adjustment for other important prognostic factors. There was no difference in survival or in the local recurrences (regardless of the preoperative radiation therapy) in a series of over 1,000 patients (Holm *et al*., 1995).

How to explain such a disparity in the outcomes? The most probable explanation, when comparing APER and SSR, is that the two operations are not interchangeable because generally they do not apply to the same type of tumors. From the clinical point of view every surgeon knows that tumors which cannot be satisfactorily removed with a SSR (very distal tumors, big tumors, low pelvis-volume/tumor-volume ratio, tethered tumors, obese, preferably male patients), are treated with an APER, and sometimes for these reasons a planned SSR is intraoperatively abandoned, and converted to an APER.

TABLE 20.1. Survival after abdominoperineal resection and sphincter-saving resection.

AUTHOR	OPERATION	N° pts	5-year SURVIVAL	P
Konn *et al.* (1993)	APER	36	80.4%	NS
	SSR	86	79.7%	
Huguier *et al.* (1997)	APER	76	43%	NS[a]
	SSR	43	43%	
Park *et al*. (1999)	APER	512	64.2%	0.018[b]
	SSR	432	71.2%	
Stocchi *et al.* (2001)	APER	309	55%	NS
	SSR	366	62%	
Law and Chu (2004)	APER	69	60.1%	0.041[a]
	SSR	419	74%	
Wibe *et al*. (2004)	APER	821	55%	0.001[a]
	SSR	1,315	68%	
Marr *et al.* (2005)	APER	190	52.3%	0.003
	SSR	371	65.8%	
Nagtegaal *et al*. (2005)	APER	373	38.5%	0.008
	SSR	846	57.6	
Chuwa and Seow-Choen (2006)	APER	93	80 mos (median)	NS[a]
	SSR	667	35-–121 mos (median)	

[a]Data confirmed at the multivariate analysis.
[b]Data not confirmed at the multivariate analysis.

TABLE 21.2. Five-year local recurrence after abdominoperineal resection and sphincter-saving resection.

AUTHOR	OPERATION	N° PTS	RECURRENCE (%)	P
Konn *et al*. (1993)	APER	36	11.4	NS
	SSR	86	10.7	
Holm *et al*. (1995)	APER	664	28	NS
	SSR	470	24	
Huguier *et al*. (1997)	APER	76	27	NS[a]
	SSR	43	34	
Stocchi *et al*. (2001)	APER	309	18	NS
	SSR	364	14	
Kapiteijn *et al*. (2001)	APER	480	4.9–10.1	NS[b]
	SSR	1180	1.2–7.3	
Killingback *et al*. (2001)	APER	58	12.6	NS
	SSR	468	7	
Law and Chu (2004)	APER	69	23.5	=0.01[c]
	SSR	419	10.2	
Wibe *et al*. (2004)	APER	821	15	0.0008[c]
	SSR	1315	10	
Chiappa *et al*. (2005)	APER	61	11	0.007
	SSR	92	39	
Marr *et al*. (2005)	APER	190	23.8	0.002
	SSR	371	13.5	
Chuwa and Seow-Choen (2006)	APER	93	5.4	NS[a]
	SSR	477	3.7	

[a]Data confirmed at the multivariate analysis.
[b]Lower values are for pts receiving preoperative Resonance Tomography (data at 2 years).
[c]No difference after adjustment for prognostic factors.

POTENTIAL PROGNOSTIC FACTORS DIFFERENTIATING APER and SSR

The prognosis of the rectal cancer amenable to surgical resection is affected by several factors that include: the site of the lesion and the distance from the anal verge, the stage of the disease and the age of the patient, the involvement of the circumferential margin and the tumor or bowel perforation during the operation.

A. Site of the tumor in the rectum

Even if *ad hoc* investigations have not consistently confirmed a better quality of life of patients retaining the sphincterial function compared to those with a permanent colostomy, it is quite common that the vast majority of the patients requiring surgery for a rectal cancer be concerned for their anal function. As a consequence, a common practice is to attempt to save the sphincters if this is compatible with a microscopically radical (R0) resection.

Since the more distal the tumor, the more difficult and demanding the sphincter-saving procedure, it is expected that the median level of the tumor is different in patients submitted to APER than in SSR.

According to the data available in the recent literature comparing the two procedures (Chiappa *et al*., 2005; Chuwa and

Seow-Choen, 2005; Holm *et al*., 2001; Nagtegaal *et al*., 2002; Wibe *et al*., 2004), tumors treated with APER are significantly more distal (1–3 cm) than those requiring a SSR.

Site of the tumor, *per se*, may have a prognostic relevance. A multivariate analysis by Freedman *et al*. (1984) determined that height of the tumor, stratified at 0–5, 6–8, 9–15 cm, from the anus, independent from other factors, influenced survival of curatively resected patients. These analyses confirmed previous observations by many authors (Glenn and MsSherry, 1966; McDermott *et al*., 1985; Moosa *et al*., 1975). More recently, Wibe *et al*. (2004) using a similar classification of the tumor level in 2,136 cases, confirmed that height of the tumor significantly affected both local recurrence and survival at the univariate and multivariate analysis. Also, Phang *et al*. (2002) reported that distance from the anus affected survival in curative stages I and II and, after the adjustment for stage, affected overall (but not local) recurrence.

Hermanek *et al*. (1989) found at the multivariate analysis that tumors of the lower third had a relative risk = 2.6 for survival when compared with upper and mid-rectum tumors.

Park *et al*. (1999) also reported a worse prognosis for low-lying tumors but difference only approximated the statistical significance.

Data regarding the association between the site of the primary and frequency of local recurrences are summarized in Table 21.3.

Bentzen *et al*. (1992) showed that the distance from the anal verge had a significant influence on the time in local

TABLE 21.3. Site of tumor/distance from anal verge and local recurrence.

AUTHOR	SITE OF TUMOR/DISTANCE FROM ANAL VERGE (cm)	5-YEAR RECURRENCE (%)	P
Kapiteijn *et al*. (2001)	< 5	10 (1.3)[a,b]	0.02
	5–10	10.1 (1)[c]	
	> 10	3.8 (5.8)[c]	
Holm *et al*. (2001)	< 6	24.8	NS
	6–10	18.6	0.03
	> 10	13.4	0.01
McDermott *et al*. (1985)	Low	26	0.001
	Middle	21	
	Upper	14	
Killingback *et al*. (2001)	> 12	4	0.04[d]
	< 12	8.9	
Bonadeo *et al*. (2001)	Low	17.9	
	Middle	7.1	0.002[a]
	Upper	5.1	
Wibe *et al*. (2004)	Low	15	
	Middle	13	0.001
	Upper	9	

[a]Multivariate analysis showed a HR = 2.98 for low tumors.
[b]Significant at multivariate analysis.
[c]pts treated with preoperative Resonance Tomography.
[d]Difference not significant after adjusting for stage.

recurrence in both Dukes' B and C stages and Abulafi and Williams in a extensive review, quoting six articles (Stearms and Binkley 1953, Morson et al. 1975, Vandertoll and Beahrs 1965, Piliphsen *et al*. 1984 and Bentzen *et al*. 1992) concluded: "*Thus, there is a general unanimity about the prognostic significance of the tumor level in the rectum. It has been suggested that the higher recurrence rate for low rectal lesions may be due to the limited space in the lower pelvis, permitting readier spread of the tumor to contiguous tissue and so making it more difficult to eradicate completely*."

More recent data show that 5-year local recurrence rate for distal cancer was always equal to or, more often, higher than 10% regardless of a preoperative treatment with radiation (Kapiteijn *et al*., 2001). At a multivariate analysis it was estimated to be 1.5–3 times more frequent than for proximal tumors (Bonadeo *et al*., 2001; Eriksen *et al*., 2007; Kapiteijn *et al*., 2001). Law and Chu (2004) also found in a multivariate analysis that distal site of the tumor was an independent risk factor for a higher local recurrence rate.

In a large study of curative resections for rectal cancer from the Cleveland Clinic Foundation, in which TME was limited to lower and middle third tumors, the rate of local recurrence alone was 2.8% for tumors above 10 cm and 8.6% for those below 10 cm (Lopez-Kostner *et al*. 1998).

These findings are not surprising because distal tumors have been associated with other adverse prognostic determinants: they include statistically more frequent advanced stages (Wibe *et al*., 2004) or N+ (Andreola *et al*., 1996) or T4 classes (Wibe *et al*., 2004), and lesions which are poorly differentiated (Wibe *et al*., 2004) or involve the circumferential resection margin (Wibe *et al*., 2004; Nagtegaal *et al*., 2002).

Moreover, bowel or tumor perforation during resection occurred in 12.5–13% of the resections at a lower level and in 7.7–8% at the intermediate level and 4.9–5% at the upper level (Wibe *et al*., 2004) ($p = 0.0001$). However, when analysed in a multivariate model, the effect of tumour level on the risk of perforation disappeared. Eriksen *et al*. (2004) demonstrated that the rate of perforation was three times higher for T4 tumors compared with T3 tumors. T4 tumors, with invasion of neighbouring organs, are an obvious challenge to the surgeon with regard to *en bloc* resection of the tumors and adjacent organs and structures.

B. Distance from the anal verge

Data are summarized in Table 21.4 and confirm that tumors requiring an APER were placed more distally (average 2 cm or more) compared with those operated on with the conservative surgery.

In the experience of Wibe *et al*. (2004) 82% of the APER were performed for tumors placed at 0–5 cm from the anus, whereas only 9% of the SSR were done for tumors lying at that level ($p < 0.001$).

C. Stage and age

It is noteworthy that T4 tumors accounted for a higher proportion (14% versus 6%, $p < 0.001$) of patients submitted to APER or to SSR, respectively (Wibe *et al*., 2004), and patients with total rectal excision were also older (Wibe *et al*., 2004).

D. Circumferential resection margin

Table 21.5 shows that a positive circumferential margin occurred after APER with a frequency 2–3 times higher than after SSR.

TABLE 21.4. Distance of the tumor from anal verge in abdominoperineal resection and sphincter-saving resection.

AUTHOR	OPERATION	N° PTS	DISTANCE (cm)	P
Holm *et al*. (1995)	APER	516	11, median	
	SSR	776	5, median	
Chiappa *et al*. (2005)	APER	61	2.4, mean	< 0.001
	SSR	92	4.9, mean	
Nagtegaal *et al*. (2005)	APER	373	< 5 (82%)	
	SSR	864	< 5 (8.9%)	
Chuwa and Seow-Choen (2006)	APER	93	1, median	< 0.001
	SSR	477	5–7, median	

TABLE 21.5. Frequency of positive/incomplete circumferential margin (CRM) after abdominoperineal and sphincter-saving resection.

AUTHOR	OPERATION	N° PTS	POS/Inc. CMR (%)	P
Wibe *et al*. (2004)	APER	721	12	< 0.001
	SSR	1315	5	
Nagtegaal *et al*. (2002)	APER	205	28.8	0.001
	SSR	451	13.5	

E. Tumor/bowel perforation

The high rate of perforation during APER was initially reported by Zirngibl *et al*., (1990) who found a sixfold greater incidence of perforation during APER than during SSR (12.9% versus 2.2%).

Tumor or bowel perforation was 4–5 times more frequent (16% versus 4% and 13.7% versus 2.5%) in APER and SSR, respectively (Wibe *et al*., 2004; Nagtegaal *et al*., 2002). Eriksen *et al*. (2004) found at a multivariate regression analysis that risk of perforation was significantly greater during APER (OR = 5.4). It has to be noted, however, that it is important to determine whether the perforation occurred in the tumor or in the adjacent bowel (Slanetz *et al*.,1984) because only the rupture of the tumor would be relevant to the outcome.

Other Factors

In the experience of Holm *et al*. (1995) men are treated by APER more often than women (p = 0.004), as tumors are larger than 5 cm in the former (p = 0.002). Furthermore surgery was considered curative more often in patients treated with SSR than in those treated with APER (p = 0.003). Nagtegaal *et al*. (2005) also confirmed that tumors removed by an APER were significantly bigger (4.6 versus 4.2 cm) than tumors resected with a SSR.

TECHNICAL ASPECTS OF THE APER

The above-mentioned data show that outcome after APER may be worse than after SSR even if data from literature are

quite inconsistent. It is true, however, that APER is generally performed for lesions which are associated with poor prognostic indexes. The majority of these indexes are tumor-dependent and only a few of them are surgery-dependent.

First, we have to consider that APER is performed more often for large and distal tumors, and site, *per se*, has a prognostic relevance in multivariate analysis since the more proximal to the anus the tumor is, the higher the risk for a short survival and for the occurrence of local relapse.

Distal tumors also present more frequently in advanced stages. Surgery-dependent prognostic factors include the higher prevalence of tumor or bowel perforation in distal tumors and in those treated with APER. It is however true that bowel or tumor perforation occurs more frequently in T4 patients which account for a higher proportion of distal lesions or lesions requiring APER.

The significance of a positive circumferential margin is less clear because it might indicate a reduced surgical volume around the mesorectum or, more simply, the lack of adequate space, being the tumor-containing distal rectum constrained within a narrow pelvis. The recent report (Nagtegaal *et al*. 2005) which showed that one third of cases submitted to APER have the resection margin in the lumen or in the submucosa, or sphincters rather than on the surface of the sphincters (or wider using a complete levator ani excision), which suggests that a poor quality of the performance of the APER can also account for a high rate of local failure.

In conclusion, although surgeons resort to perform an APER (this is often an intraoperative decision) when there are many oncologically negative prognostic factors which preclude a low or a ultralow anastomosis nevertheless, APER should be done in the best possible way.

Herewith we report the essential steps for an adequate APER following the standard approach in open surgery and we emphasize the fact that the perineal surgeon must be experienced in the technique of APER.

We think that the procedure used by the pelvic surgeon is extremely simplified if he/she intervenes in a late phase of the rectal mobilization, when the abdominal surgeon has isolated circumferentially the block of the rectum and mesorectum at the level of the plane of the levator ani. Care should be paid to avoid any dissection of the mesorectum from the levator ani; this muscle, together with the mesorectum and the rectum, should be resected in block mainly during the perineal phase (Holm *et al*., 2007). The abdominal surgeon should proceed just in front of the sacrum, to the coccyx, using a diathermy dissection that simplifies the haemostasis in the poorly accessible areas and might minimize the development of local recurrence. This is important because it has been reported that 62% of perforations occur at this stage (Porter *et al*., 1996).

Perineal Phase

The patient should be properly positioned on the table with the perineum over the edge of the bed and the sacrum raised by a pad. The shoulders should be adequately supported to avoid the patient slipping down during the Trendelenburg position. The perineal operator should irrigate the rectum with a povidone-iodine solution to clear secretions, blood, and loose bits of tumor. Next, the anus is closed tightly with a heavy anal purse-string, and then the perineal area is sterilely prepped.

Because the exposure is paramount, it may be useful to tape (or suture) the buttocks laterally should the surgeon face with heavy buttocks or a deep anal canal, which make visualization difficult. A headlight is valuable. A Foley catheter may be inserted to guide the operator during the anterior dissection.

Incision

A wide elliptical skin incision is made with cutting cautery extending ventrally, from the mid portion of the perineal body in males or the posterior aspect of the vaginal introitus in females, to the tip of the coccyx posteriorly. The ellipsis should be wide enough to encompass the anal sphincter muscle complex. If vaginectomy is planned, the elliptical incision is prolonged anteriorly to incorporate the posterior part of the vagina. The edges of the perineal ellipse may be sutured to further close the anal canal.

Then grasp this suture with two Kocher to provide a convenient handle for traction on the rectum.

After division of the superficial fascia between the subcutaneous tissue and the ischiorectal fat, place a self-retractor (i.e., St Mark retractor, Lone Star or Gelpi) to facilitate subsequent dissection. The initial incision is deepened with the cautery down through the fat of the ischiorectal fossae to the level of the levator ani. It is important that the dissection be maintained outside the subcutaneous portion of the external anal sphincter.

Hemostasis is provided by coagulation or suture ligation of the inferior rectal vessels.

Posterior Dissection

The surgeon palpates the tip of the coccyx to guide the direction of the initial posterior dissection and proceeds by exposing its ventral aspect and dividing the anal coccigeal ligament which also releases the attachments of the superficial external sphincter muscle. After entering the superficial postanal space, the index of the perineal operator can feel the finger of the abdominal surgeon: they are only separated by the dense Waldeyer fascia. This needs to be sharply divided, in a transverse manner, by pushing the scissor just in front of the coccyx and spreading it to widen the gap to allow the index to enter in the true pelvis and find the finger of the abdominal operator. During this maneuver the perianal skin ellipse is retracted ventrally to elevate the anal canal and the rectum which can be maintained ventrally with the help of a narrow malleable retractor bent in an L shape.

It is important to avoid two mistakes: to dissect too posteriorly and to lift the presacral fascia from the sacrum which could result in disruption of the presacral venous plexus and life-threatening bleeding, and to remain too anteriorly and violate the integrity of the most caudal part of the mesorectum. For this reason the perineal surgeon should proceed with this dissection only after the abdominal surgeon has completely isolated the rectum at the level of the levator ani and can displace it far from the sacrum and coccyx with a St Mark retractor.

Should this maneuver be difficult or impossible, this means that the tumor is extending posteriorly and this, more than an erroneous perineal procedure, may be the cause for a subsequent local oncologic failure.

Lateral Dissection

The perineal surgeon proceeds dividing the ischiorectal adipose tissue on both

sides, close to the lateral pelvic walls and gaining a direct vision with the use of hand-held malleable narrow retractor. Through the dorsal defect previously created, the index finger is inserted into the pelvis, along the pelvic sidewall to hook the iliococcygeous muscle which is divided with electrocautery and then the surgeon hooks the pubococcygeal muscle which is similarly divided or transfixed with a 2-0 absorbable suture ligature. If there is any residual Waldeyer fascia remaining on the lateral aspects, it should be divided. The ischial spines define the lateral extent of the dissection.

The dissection should proceed circumferentially to isolate all the posterior and lateral two-third of the anorectum. When the posterolateral aspect of the rectum is freed and fully mobilized, and there is enough space, the specimen including the resected rectosigmoid is delivered from the abdominal cavity through the perineal defect. The rectum is then turned over in a U shape on itself to facilitate the subsequent anterior dissection.

Anterior Dissection

The procedure starts by widening the space between the anterior skin, which is retracted upward, and the anorectum, which is displaced downward. After dissection of the subcutaneous tissue the surgeon proceeds by separating the anterior decussating fibers of the external sphincter until the superficial transverse perineal muscle is identified. It is important to be aware that anteriorly there is no plane through which the dissection can be performed bluntly, and to avoid injury to the membranous urethra, one should remain dorsally to the bulbocavernous muscle. To make this procedure easier the following points seem relevant.

Firstly, the abdominal surgeon should have already mobilized the rectum between the rectal fascia propria and the Denonvilliers fascia, and the dissection should be developed to seminal vesicles and the inferior margin of the prostate, which then can be displaced anteriorly through a St Mark retractor.

Secondly, intermittent palpation of the Foley catheter may assist in keeping the dissection along the safe plane, and this is the main reason for using, at least temporarily, a urethral catheter instead of percutaneous suprapubic one.

Thirdly, the angle of dissection to detach the anal canal is directed upward toward the pubic bone. Then the angle is (in the midline), directed toward the sacral promontory, because the rectum is sharply angulated owing to the U-shaped puborectalis sling (the attachments of rectourethralis and puborectalis muscles).

Fourthly, the dissection may be safer if it starts from the side rather then from the midline. In fact, displacing the rectum to one side places the puborectalis muscle under tension, exposes the groove between the lateral edge of the prostate and the rectum, and allows the surgeon to carefully sever the puborectalis from the side with electrocautery. This has to be performed bilaterally to gradually twins the median attachments (fibers of the rectourethralis muscles) and the fibrous bands on the lateral aspects of the prostate. The surgeon supports with his left hand the anorectum and then divides sharply to avoid any avulsion from the urethra and the prostate capsule.

CONCLUSION

In conclusion, it is clear that the distal localization of the rectal tumor raises many problems with its radical resection through the APER which, however, is the only available surgical option for a low-lying malignancy. Besides the fact that stage is often advanced in patients receiving an APER, there are two additional anatomical conditions, as emphasized elsewhere (Bozzetti, 2006), which increase the risk of local recurrence with such operation: (1) the lack of an anatomical plane of dissection, especially in the ventral perineum, which obliges the surgeon to find a compromise between the optimal tumoral clearance and the desire of avoiding any injury to the genitourinary tract; (2) the distal rectum is surrounded, especially in the anterior two thirds of its circumference, by such a thin layer of perirectal fat, that it is very easy to remove the circumferential margin and then the line of transection falls in neoplastic tissue.

This may explain why SSR is more often considered curative than APER as already reported by Holm *et al*. (1995) more than 12 years ago.

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22
Chemoradiation for Rectal Cancer

Max Mano and Jean-Pascal Machiels

INTRODUCTION

Rectal cancer is a complex disease in that it is associated not only with a significant risk of systemic spread (similar to colon cancer) but also of loco-regional extension and complications. Loco-regional failures can be occasionally salvaged by surgery, but the prognosis of these patients remains poor. Furthermore, inoperable local recurrences or persistent disease can severely impair patients' quality of life, reinforcing the importance of aggressive loco-regional therapy. Early multidisciplinary consultation, coordinated multimodality treatment and physician's experience are considered key tools to achieve excellence in the management of this disease.

Recent improvements in the management of rectal cancer reflect progress made over several fronts. The widespread availability of endoscopic ultrasound and magnetic resonance imaging has dramatically improved our knowledge of tumor anatomic relations and extension of lymph node spread, thereby facilitating treatment decisions. Radiotherapy techniques have also improved over time, particularly in terms of limiting irradiation of normal surrounding structures and optimizing the delivery of radiation to the tumor bed and regional lymph nodes. Simplified and less toxic chemotherapy schedules have also facilitated the delivery of chemoradiation. Finally, further improvement in local control rates has been achieved with the advent of total mesorectal excision. One of the greatest challenges today is how to best integrate all of these modalities in daily clinical practice.

APPLICATIONS OF PRE- OR POSTOPERATIVE CHEMORADIATION

In old institutional series and clinical trials, in which patients were treated with what is now considered suboptimal surgery and radiotherapy techniques, loco-regional failure rates were as high as 1/3 (Swedish Rectal Cancer Trial, 1997; Havenga *et al*., 1999; Phillips *et al*., 1984; Kapiteijn *et al*., 1998). With the advent of total mesorectal excision and the growing use of preoperative radiotherapy or chemoradiation for locally advanced disease, these figures have gradually decreased to achieve rates

of 5% or less (Sebag-Montefiore *et al*., 2006; Kapiteijin *et al*., 2001). The combination of radiotherapy and chemotherapy as a means of increasing tumor cell kill, mainly by enhancement of radiosensitivity, has been proven a successful concept in many solid tumors such as squamous cell carcinomas of the head and neck and oesophagus, anal cancer, and adenocarcinomas of oesophagus, stomach and pancreas. Preclinical data supported the validity of this concept in rectal cancer (Byfield, 1989), providing the rationale for the investigation of chemoradiation in the clinical setting.

Because of the significant risk of loco-regional failure, pelvic radiation is routinely used in patients with stage II or III rectal cancer. Both pre- and postoperative radiotherapy are known to decrease local relapse rates (Colorectal Cancer Collaborative Group, 2001). In the US, postoperative 5-fluorouracil based chemoradiation was recommended (NIH Consensus Conference, 1990) after the Gastrointestinal Tumor Study Group demonstrated that chemoradiation, but not chemotherapy or radiotherapy alone, improved the outcome of patients operated from stage II or III rectal cancer (Gastrointestinal Tumor Study Group, 1985). A similar improvement in disease-free and overall survival (but also in local control rates) was reported with chemoradiation in the NCCTG trial, which compared adjuvant radiotherapy to chemoradiation with 5-fluorouracil-based chemotherapy (Krook *et al*., 1991). Finally, NSABP R02 confirmed the importance of postoperative chemoradiation, which was able to reduce local recurrence rates from 13% to 8% when compared to chemotherapy alone (Wolmark *et al*., 2000). In Europe, where the treatment is usually administered preoperatively, the role of radiotherapy was established in the mid 1990s after the Swedish trial showed a significant improvement in local control and also survival with preoperative radiotherapy. Even if the EORTC 22931 trial failed to confirm the superiority of preoperative chemoradiation over radiotherapy alone in term of survival and disease-free survival (Bosset *et al*., 2006), preoperative chemoradiation has been usually accepted as standard treatment for stage II and III rectal cancer in Europe.

In short, for stage I (T1-T2N0M0) rectal cancer, upfront surgery remains the standard of care, with postoperative radiotherapy or chemoradiation offered to occasional patients who will be upstaged to pathological stage II or III. Stage IIA (T3N0M0) is generally considered an indication for preoperative treatment. However, the risk of local relapse is known to be low in high-lying T3N0 tumors with negative circumferential margins after total mesorectal excision, so that some stage IIA patients may not benefit from radiotherapy. Magnetic Resonance Imaging can now predict with good accuracy the status of circumferential margins (Mercury Study Group, 2006) and assist physicians with this decision, though this approach has not been prospectively validated. For stage IIB (T4N0M0) and beyond (node positive), there are good arguments to support the use of preoperative chemoradiation, with postoperative systemic chemotherapy often indicated as an attempt to reduce distant recurrence rates.

PREOPERATIVE VERSUS POSTOPERATIVE CHEMORADIATION

Although the role of radiotherapy or chemoradiation is well established in stages II and III rectal cancer, it has been a matter of debate whether it should be done pre- or postoperatively. Based on current evidence, preoperative treatment has been advocated and has been increasingly accepted as standard of care, even in North America where the postoperative approach had been traditionally preferred (NIH Consensus Conference, 1990). The most important clinical trials comparing preoperative to postoperative treatment are listed in Table 22.1. Theoretically, the potential advantage of postoperative treatment is a more accurate pathological staging, with chemoradiation delivered only to patients with pT3-4 and/or node positive, thereby reducing the risk of overtreatment. One potential application for this concept are the data reported by two North American institutions who identified a favorable subset of patients (well to moderately differentiated histology, no lymphovascular invasion, extending ≥2 mm into the perirectal fat) with T3N0 disease that may not require adjuvant therapy (Willett *et al*., 1999; Merchant *et al*., 1999). Furthermore, in the Dutch trial, patients with T3N0 disease had a low incidence of 2-year local failure (5.7%), as did patients with primary tumors 10–15 cm from the anal verge (4%) (Kapiteijn *et al*., 2001).

The two North American trials (INT 0147 and NSABP R-03) that compared preoperative to postoperative chemoradiation were prematurely closed because of slow accrual. Results of NSABP R-03 suggested a non-significant but clinically meaningful improvement in overall and disease-free survival as well as higher sphincter preservation rates with preoperative treatment (Roh *et al*., 2004). The best evidence supporting the superiority of preoperative over postoperative treatment is based on the German trial in which 823

TABLE 22.1. Preoperative (chemo)radiation is the standard of care.

	N	Treatment	G3/4 toxicity (%)	Local failure (%)	Survival (%)
Swedish (Folkesson *et al*., 2005)	1,168	Surgery		26 (13-y)	30 (13-y)
		RT + surgery		9*	38*
Dutch (Kapiteijin *et al*., 2001; Marijnen *et al*., 2004)	1,011	Surgery		11.4 (5-y)	82 (2-y)
		RT + surgery		5.8*	82
German (Sauer *et al*., 2004)	823	CRT → surgery	27*	6* (5-y)	76 (5-y)
		Surgery → CRT	40	13	74
MRC-07 (Sebag-Montefiore *et al*., 2006)	1,350	RT → surgery		4.7* (3-y)	80.8 (3-y)
		Surgery → selective[a] RT		11.1	78.7

N = number of patients; CRT = chemoradiation; RT = radiotherapy; G = grade; y = year.
*$p < 0.05$.
[a]Recommended to patients with postoperative involved circumferential margins.

patients with stage II or III rectal cancer were randomized to receive either pre- or postoperative chemoradiation (5,040 cGy/FU 1,000 mg/m^2 during the first and fifth weeks of radiotherapy) followed by 4 months of adjuvant systemic chemotherapy in both arms (Sauer *et al*., 2004). All patients were expected to undergo total mesorectal excision. Although there were no significant differences in overall, disease-free or distant disease-free survival, sphincter-preserving surgery and local control rates were significantly increased by preoperative treatment administration (39% vs. 19%; p = 0.004 and 94% vs. 87%; p = 0.006, respectively; Table 22.1). In addition, acute and late bowel toxicity was lower in the preoperative treatment arm. One of the strengths of the study is the fact that the treatment was identical in both arms, such that supporters of preoperative chemoradiation now consider this study a 'proof-of-principal'.

MCR CR-07 was a pragmatic trial that addressed the question of potential overtreatment with the systematic use of preoperative treatment (Sebag-Montefiore *et al*., 2006). One thousand three hundred-fifty patients with rectal cancer (< 15 cm for the anal margin) were randomized to receive either preoperative short course radiotherapy (25 Gy in five fractions) followed by surgery or upfront surgery followed by selective postoperative chemoradiation (45 Gy in 25 fractions + infusional 5-fluorouracil) for patients at higher local risk (involved circumferential margins in most cases). Patients were given adjuvant chemotherapy as per local practice. The results showed a significant reduction in local recurrence rates with preoperative radiotherapy (4.7% vs. 11.1% at 3 years; 95% CI, HR = 2.47 [1.61–3.79]; Table 22.1). There were no significant differences in disease-free or overall survival. This important trial further supports the superiority of preoperative over postoperative treatment, which was radiotherapy alone in this case.

PREOPERATIVE RADIATION ALONE VERSUS CHEMORADIATION

There is a large body of evidence supporting preoperative instead of postoperative treatment administration, but the importance of adding chemotherapy to radiotherapy has never been properly evaluated in the preoperative setting particularly after the demonstration of the critical role of total mesorectal excision in improving local control rates. The role of preoperative radiotherapy in improving local control rates is supported by the weight of a meta-analysis of randomized clinical trials that compared preoperative (14 trials, n = 6,350) or postoperative (8 trials, n = 2,157) radiotherapy to surgery alone prior to the introduction of total mesorectal excision. The reduction in the 5-year rate of local recurrence was more pronounced in the preoperative radiotherapy trials (22.2% with surgery alone to 12.5% [p < 0.00001]) than in the postoperative radiotherapy trials (22.9% with surgery to 15.3% [p = 0.0002]) (Colorectal Cancer Collaborative Group, 2001). Another meta-analysis, which included only randomized clinical trials comparing preoperative radiotherapy followed by surgery to surgery alone, confirmed the significant impact of preoperative radiotherapy in reducing local recurrences (OR, 0.49; 95% CI, 0.38–0.62; P < 0.001) and also suggested a small survival benefit (Cammà *et al*., 2000). Also

in the pre-total mesorectal excision era, a Swedish trial demonstrated the value of preoperative radiotherapy in improving local control rates and even suggested a significant survival benefit (Swedish Rectal Cancer Trial, 1997). However, local recurrence rates in the surgery alone arm in the Swedish trial were unacceptably high for post-total mesorectal excision standards, which led the Dutch to perform a similar randomized trial assessing the role of preoperative radiotherapy followed by total mesorectal excision (Kapiteijin *et al*., 2001). The results of this trial confirmed (1) the importance of total mesorectal excision, which alone reduced local recurrences rates to close to 10% and (2) the importance of preoperative radiotherapy even when total mesorectal excision was performed (5-year cumulative local recurrence rates 11.4–5.8%) (Marijnen *et al*., 2004). In MRC CR-07 (Quirke *et al*., 2006), discussed in detail above, approximately 50% of the patients did not undergo mesorectal plane surgery resulting in local recurrence rates in the range of 6–9% even after preoperative radiotherapy. However, in the subgroup of patients who underwent total mesorectal excision, the local recurrence rate in the preoperative radiotherapy arm was as low as 1%. In this trial, there was also a significant improvement in sphincter preservation rates, which is sometimes a concern with the short course schedule of preoperative radiotherapy. These results are in line with those of the Dutch trial and are indicative of the optimal rates of local control achieved with preoperative radiotherapy alone in the setting of optimal surgery.

Subset analysis of some clinical trials have suggested a correlation between patient outcome and the achievement of pathological complete response (Roh *et al*., 2004; Rödel *et al*., 2005; Rosenthal *et al*., 2003; Ryan *et al*., 2006), which is much more commonly seen with chemoradiation than with radiotherapy alone. These data support the concept of combining chemotherapy and radiotherapy in patients with locally advanced rectal cancer. Another trial that attempted to address the role of chemoradiation in the preoperative setting was EORTC 22921 (Bosset *et al*., 2006). In this study, 1,011 patients with T3 or T4 rectal cancer were randomized to one of four arms: preoperative radiotherapy, preoperative chemoradiation, preoperative radiotherapy and postoperative chemotherapy, or preoperative chemoradiation and postoperative chemotherapy. Radiotherapy consisted of 45 Gy delivered over a period of 5 weeks and chemotherapy consisted of 5-fluorouracil and leucovorin for two cycles during the first and fifth weeks of radiotherapy. The primary endpoint was overall survival. There were no significant survival differences after 5 years of follow-up, but the trial was probably underpowered for this endpoint. Most importantly, the cumulative rates of local recurrence ranged from 7.6% to 9.6% in the three groups that received chemotherapy at any point, versus 17.1% in the group that did not receive any chemotherapy ($p = 0.002$), suggesting that the latter may have a role in improving local control rates. The interpretation of the results of this trial is difficult in several aspects. Total mesorectal excision was recommended only 6 years after the trial had been started. Roughly one third of the patients are known to have had total mesorectal excision but in up to 50% the type of resection is unknown. As shown in Table 22.2, preoperative chemoradiation resulted in higher downstaging

TABLE 22.2. Preoperative chemoradiation versus radiotherapy alone.

	N	TME surgery rates	Regimen	pCR (%)	Local failure (%)	5-y DFS (%)	5-y survival (%)
FFCD 9203 (Gerard *et al*., 2006)	762	Probably only a minority of patients	RT → p.o. CT	3.7*	16.5	56	66
			RT + FU → p.o. CT	11.7	8	59	67
EORTC22921 (Bosset *et al*., 2006)	1,011	At least one third of the patients[a]	RT	5*	17.1	54	65
			RT → p.o. CT		9.6*		
			RT + FU	11	8.8*	56	66
			RT + FU → p.o. CT		8*		

N = number of patients; pCR = pathologic complete response; DFS = disease-free survival; y = year; FU = 5-fluorouracil; RT; radiotherapy; CT = chemotherapy; p.o. = postoperative; EORTC = European Organisation for the Research and Treatment of Cancer; FFCD = Féderation Française de Cancérologie Digestive.

*$p < 0.05$.

[a]Type of surgery unknown in up to 50% of the patients.

and pathological complete response (11% versus 5%, respectively) as compared to radiotherapy alone but rates of sphincter-preserving surgery were not different (50.5% vs. 52.8%). Rates of postoperative complications and late side effects were similar. In short, this study failed to confirm that preoperative chemoradiation improves local control or sphincter preservation rates as compared to radiotherapy alone, provided that chemotherapy is given as adjuvant treatment.

The French trial FFCD 9203 also compared preoperative 5-fluorouracil-based chemoradiation to radiation alone in patients with T3/T4Nx rectal cancer (Gerard *et al*., 2006). Again, no survival difference was observed, but the 5-year incidence of local recurrence was lower in the chemoradiation arm (8.1% vs. 16.5%; $P < 0.05$). Pathological complete response and toxicity were increased by chemoradiation, but sphincter preservation rates were similar (Table 22.2). In this study, adjuvant chemotherapy was given to all patients. As a note of caution, total mesorectal excision was not routinely performed in this trial.

In summary, the expected level I evidence supporting the superiority of preoperative chemoradiation over radiotherapy alone is still lacking. Clinical trials specifically addressing this question have been plagued by technical problems. With modern surgery and radiotherapy, loco-regional failure rates appear to be extremely low (usually around or below 5%) (Quirke *et al*., 2006; Kapiteijin *et al*., 2001). However, it should be pointed out that these trials have included unselected rectal cancer patients, and it is possible that subgroups of patients at higher local risk (e.g., bulky T4, node positive, low-lying tumors) may derive a selective benefit from the addition of chemotherapy to

preoperative radiotherapy, particularly in terms of local control and sphincter preservation. The demonstration of increased downstaging with the use of chemoradiation, as compared to radiotherapy alone in the EORTC trial supports this concept (Bosset *et al*., 2006). In the clinical practice, a growing number of institutions have considered preoperative chemoradiation standard of care for patients with stage II or III rectal cancer, based on the evidence above and on the data available from postoperative chemoradiation trials.

INTEGRATION OF NEW CYTOTOXIC AND BIOLOGICAL AGENTS IN THE PREOPERATIVE SETTING

In the early 1990s, INT 864751 demonstrated that the protracted 5-fluorouracil infusion throughout the postoperative radiotherapy improved relapse-free and overall survival as compared to bolus 5-fluorouracil given during the first and fifth weeks of radiotherapy, but there was no advantage from adding semustine to the chemotherapy schedule (O'Connell *et al*., 1994). Using the best INT 864751 arm as control, INT 0114 evaluated two different schedules of chemotherapy in combination with adjuvant radiotherapy. In this larger trial, the schedule of 5-fluorouracil (protracted infusion or bolus) had no impact on treatment efficacy (Smalley *et al*., 2006) but, as expected, bolus 5-fluorouracil was more myelotoxic. In INT 0114, a study that compared three biochemically modulated schedules of 5-fluorouracil, the schedule of chemotherapy had no clear influence on the efficacy of chemoradiation (Tepper *et al*., 2002). In summary, at the present time, no schedule of chemotherapy has been demonstrated superior to any other in terms of efficacy such that the choice should be based mainly on patients' preference, local availability and expertise in the management of central venous catheters and risk of toxicity.

However, the advent of new cytotoxic and targeted agents has boosted interest in the investigation of novel schedules of chemoradiation. Studies are now aiming to improve local control rates as well as survival by means of increasing radiation sensitization and optimizating chemoradiation schedules. Capecitabine, irinotecan, oxaliplatin, cetuximab, and bevacizumab are highly active in colorectal cancer and have radiosensitizing properties. Irinotecan-based chemoradiation has been addressed elsewhere in this book, so that below we discuss the current data, ongoing and future research with capecitabine, oxaliplatin and targeted therapy chemoradiation. These compounds are expected to improve the efficacy of preoperative radiotherapy in terms of local control and prevention of distant metastases.

Before moving to a more general discussion of the different components investigated nowadays, we provide a brief methodology of preoperative chemoradiation in rectal cancer.

PATIENTS AND METHODS

The general treatment plan of standard preoperative chemoradiation as well as trials investigating new chemotherapy or biological agents in this setting is described in Figure 22.1.

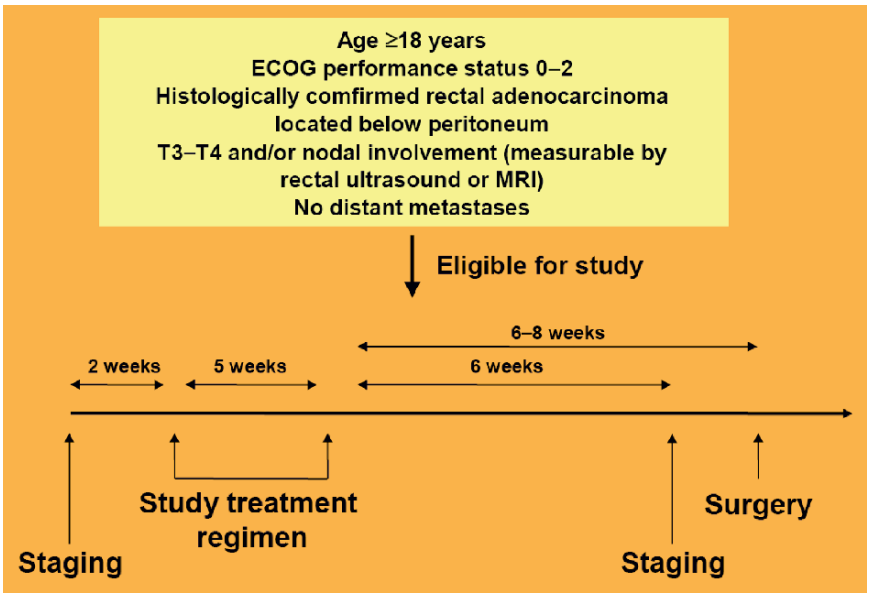

FIGURE 22.1. Treatment plan of standard preoperative chemoradiation and trials investigating new chemotherapy or biological agents.

Eligibility Criteria

General inclusion criteria were: histologically proven rectal adenocarcinoma stage T3–4 or/and N1–2 either by transrectal ultrasound or magnetic resonance imaging; age > 18 years; Eastern Cooperative Oncology Group performance (ECOG) performance status ≤ 2; and acceptable liver, renal, and hematological parameters (granulocytes > 1,500/mm^3, platelets > 100,000/mm^3, bilirubin ≤ 1 × upper limit of normal (ULN), SGOT/SGPT ≤ 2.5 × ULN, creatinine ≤1.5 mg/dl or creatinine clearance at least 60 ml/min). General exclusion criteria were: prior pelvic irradiation; active second malignancy during the previous 5 years (except nonmelanomatous skin cancer or *in situ* cervical carcinoma); pregnancy and lack of contraception; presence of any psychological, familial, sociological, or geographical condition potentially hampering compliance with the study protocol and follow-up schedule; prior or concurrent evidence of peripheral neuropathy, inflammatory bowel disease, malabsorption syndrome, synchronous colic and rectal tumors, and other uncontrolled severe disease precluding administration of chemotherapy and radiation.

Pretreatment Evaluation

Pretreatment examinations had to be performed within 4 weeks before starting treatment. Complete history and physical examination including digital rectal examination were completed, including rectoscopy with tumor biopsy, transrectal ultrasound, pelvic magnetic resonance imaging, colonoscopy, chest computed tomography, abdominal and pelvic CT, electrocardiogram, and complete laboratory tests (electrolytes, liver function, creatinine, blood urea nitrogen, creatinine

kinase enzyme, complete blood count, dosage of carcinoembryonic antigen, and pregnancy test if indicated).

Radiotherapy

Protocol of radiation therapy used in our clinical trials is given here (Machiels *et al*., 2005, 2006, 2007). Megavoltage equipment was used with 6–8-MV as minimal energy. According to the EORTC 22921 protocol, we delivered 45 Gy in 25 fractions (1.8 Gy daily from Monday through Friday, days 1–33). 3D Conformal radiotherapy was used for all patients based on a contrast computed tomography scan of the pelvis. This planning computed tomography was performed in the treatment position, with 3–5 mm thick slices. The Clinical Target Volume included the entire mesorectum, as identified by the radiologist and surgeon. Internal iliac nodes were included up to the venous bifurcation, together with the presacral nodes (limit S1/S2) (Roels *et al*., 2006). The Planning Target Volume was an isotropic expansion of the Clinical Target Volume (10 mm). Maximum, mean and median dose to the Planning Target Volume were calculated.

Chemotherapy

The scheme of one chemotherapy regimen used in our clinical trials is given here for indication (Machiels *et al*., 2005). Patients received oxaliplatin (50 mg/m^2) intravenously (i.v.) over 2 h on days 1, 8, 15, 22, and 29. Adequate hematologic parameters (neutrophil count $> 1.5 \times 10^9$/l and platelet $> 75 \times 10^9$/l) were required before each oxaliplatin infusion. Radiotherapy was performed within 2 h of oxaliplatin infusion. Capecitabine (825 mg/m^2 twice day) was given orally on each day of radiation. Patients were asked to take capecitabine as close as possible to 9.00 am and 9.00 pm. In case of adverse events, chemotherapy dose reduction was performed according to standard guidelines. History, clinical examination and laboratory tests (including renal, liver and hematologic evaluations) were performed each week during chemoradiation.

Surgery and Follow-Up

Patients underwent radical resection of rectal cancer within 6–8 weeks after completion of chemoradiation. There was no restriction of the technique used by the surgeons but total mesorectal excision was recommended and performed in all cases. Adjuvant chemotherapy was recommended in patients with nodal involvement at surgery. After surgery, all patients were followed every 3 months.

Histopathological Assessment of Response to Chemoradiation

In case of persistence of macroscopic residual tumor, standard pathological examination was performed with 3–5 sections to investigate the deepest invasion in the bowel wall. If no macroscopic tumor was present and only a small ulcer was observed, the ulcer and 2-cm periphery was examined for residual tumor and deepest invasion in the bowel wall. All lymph nodes were included according to standard procedures and the circumferential resection margin was measured according to Quirke *et al*., (1986). Pathological complete response was defined as the complete disappearance of all tumor cells. In addition, semi-quantitative evaluation of histological regression was performed according to rectal cancer regression grading established by Dworak

and colleagues: grade 0, no regression; grade 1, minimal regression (dominant tumor mass with obvious fibrosis or vasculopathy or both); grade 2, moderate regression (predominantly fibrotic changes with few tumor cells or groups); grade 3, good regression (very few tumor cells in fibrotic tissue with or without mucous substance); and grade 4, total regression (no tumor cells, only fibrotic mass). Wheeler's grade was also used: grade 1, sterilization or only microscopic foci of adenocarcinoma remaining with marked fibrosis; grade 2, marked fibrosis but macroscopic disease; grade 3, little or no fibrosis with abundant macroscopic disease.

CAPECITABINE

Capecitabine mimics continuous infusion 5-fluorouracil and has been shown effective in the treatment of colorectal cancer either in the adjuvant or metastatic settings. Capecitabine is an effective oral fluoropyrimidine that exploits the high intratumoral activity of thymidine phosphorylase to generate 5-fluorouracil preferentially within tumor tissue. In addition, radiation therapy also increases thymidine phosphorylase expression and has the potential to act synergically with capecitabine. Therefore, capecitabine may represent a more effective, better tolerated, and more convenient alternative to 5-fluorouracil for use in combination with radiotherapy. A dose-finding study suggested that capecitabine given concurrently with radiotherapy is safe and active. The recommended doses for capecitabine in combination with pelvic radiation (50.4 Gy) are (i) 825 mg/m^2 twice daily given continuously or (ii) 900 mg/m^2 twice daily given on weekdays (Monday to Friday) (Dunst *et al*., 2002; Ngan *et al*., 2004). Subsequent phase II trials have confirmed that capecitabine-based chemoradiation is safe (≤grade 3 toxicity below 10%) and effective with pathological complete response rates in the range of 4–31% (Kim *et al*., 2005). A non-randomized comparison suggested that capecitabine may lead to higher pathological complete response rates as compared to bolus 5-fluororuracil when given in combination with radiation (22% vs. 11%) (Kim *et al*., 2006). However, these results should be confirmed in large phase III trials.

OXALIPLATIN

Oxaliplatin is a third-generation platinum compound that has shown a high level of synergism with fluoropyrimidines in colorectal cancer. Results of the MOSAIC study have shown that the addition of oxaliplatin to infusional 5-fluorouracil/leucovorin (FOLFOX-4) improves disease-free survival of patients with stage II or III colon cancer (Andre *et al*., 2004). FOLFOX improves response rate and time to disease progression compared to 5-fluorouracil/leucovorin in metastatic colorectal cancer (de Gramont *et al*., 2000). Capecitabine/oxaliplatin regimens have shown similar efficacy to 5-fluorouracil/oxaliplatin in the same indication (Arkenau *et al*., 2005).

The addition of oxaliplatin to infusional 5-fluorouracil or capecitabine and concomitant radiotherapy in rectal cancer has been shown feasible and active in phase I and II studies (Ryan *et al*., 2006; Glynne-Jones *et al*., 2005). Pathological complete response rates ranging from 6% to 28% have been reported with these regimens.

The main dose-limiting toxicity is grade 3/4 diarrhea (6–33%). One recommended preoperative chemoradiation regimen for rectal cancer is capecitabine 825 mg/m^2 twice daily on days 1–14 and 22–35 plus oxaliplatin 50 mg/m^2 on days 1, 8, 22, and 29 given concurrently with radiotherapy (total dose 50.4 Gy) (Rödel *et al*., 2003).

In a phase II trial, radiation therapy (45 Gy) was combined with capecitabine (825 mg/m^2 twice daily, every day excluding weekends) and oxaliplatin (50 mg/m^2/weekly) as preoperative treatment of patients with stage II and III rectal cancer (staged by endorectal ultrasound) (Machiels *et al*., 2005). A weekly schedule of oxaliplatin was chosen to minimize the toxic effect of this drug and try to maximize its radiosensitizing properties. Pathological complete response was found in 5 of 40 patients. According to Dworak's classification, good regression (very few tumor cells in fibrotic tissue) was found in 6 (18%) additional patients. The most frequent adverse event was grade 3/4 diarrhea (30%), generally occurring during the fourth or fifth week of chemoradiation. Diarrhea was easily manageable with treatment interruption and, if necessary, dose reduction with rehydration and supportive measures as appropriate. However, diarrhea seemed to occur more frequently than with previously described chemoradiation regimens including oxaliplatin. The capecitabine, oxaliplatin, radiotherapy and excision (CORE) study investigated the same regimen and reported a 10% pathological complete response and 18% grade 3/4 diarrhea rate (Rutten *et al*., 2006). The PETACC6 trial will further investigate this new schedule in a large phase III trial. Two clinical trials investigated the feasibility of neoadjuvant chemoradiation and oxaliplatin-based adjuvant chemotherapy (Rutten *et al*., 2006; Rödel *et al*., 2007). In the Rödel study, 60% of the patients completed four cycles of adjuvant chemotherapy, and similar findings were reported in the CORE trial.

TARGETED THERAPY

Two biological agents are now widely used in combination with chemotherapy to treat patients with metastatic colorectal cancer, namely cetuximab and bevacizumab. Selected phase I/II trials combining targeted therapies with preoperative chemoradiation for rectal cancer are summarized in Table 22.3. Cetuximab is a chimeric IgG1 monoclonal antibody that binds to epidermal growth factor receptor with high specificity and with a higher affinity than either epidermal growth factor or transforming growth factor-alpha. Cetuximab is effective in the treatment of metastatic colorectal cancer as monotherapy and in combination with chemotherapy (Cunningham *et al*., 2004). In addition, cetuximab improves survival in combination with curative-intent radiotherapy in patients with locally advanced head and neck carcinoma (Bonner *et al*., 2006). Therefore, the addition of cetuximab to the preoperative chemoradiation treatment of patients with locally advanced rectal cancer may potentially improve their outcome.

A phase I/II trial investigated the safety and feasibility of concurrent radiotherapy, capecitabine and cetuximab in the preoperative treatment of patients with rectal cancer (Machiels *et al*., 2007). The recommended dose of capecitabine in combination with cetuximab (initial dose 400 mg/

TABLE 22.3. Selected phase I/II trials of preoperative chemoradiation regimens including targeted agents

Trial	No. of patients	Regimen	Grade 3/4 diarrhoea (%)	PCR (%)
Czito *et al*., 2006	6	50.4 Gy Capecitabine 650 mg/m^2 twice daily[a] Gefitinib 250 mg daily[a]	17	0
Chung *et al*., 2006	20	50.4 Gy 5-FU 225 mg/m^2 CI Cetuximab 400 mg/m^2 day 1 (250 mg/m^2 weekly thereafter)	10	12
Arnold *et al*., 2006	46	50.4 Gy Capecitabine 500 or 650 or 825[b] mg/m^2 twice daily (dose escalation) Oxaliplatin 50 mg/m^2 (days 1, 8, 22, and 29) Cetuximab 400 mg/m^2 day 1 (250 mg/m^2 weekly thereafter)	< 20	9
Hofheinz *et al*., 2006	20	50.4 Gy Capecitabine 400 or 500[b] twice daily (dose escalation) Irinotecan 40[b] or 50 mg/m^2 (days 1, 8, 15, 22, and 29) (dose escalalation) cetuximab 400 mg/m^2 day 1 (250 mg/m^2 weekly thereafter)	< 20	25
Machiels *et al*., 2007	40	45 Gy Capecitabine 650 or 825[b] mg/m^2 twice daily (dose escalation) Cetuximab 400 mg/m^2 day 1 (250 mg/m^2 weekly thereafter)	15	5
Willett *et al*., 2004	6	50.4 Gy 5-FU 225 mg/m^2 CI Bevacizumab (5[b] mg/kg every 2 weeks)	0	0
	5	50.4 Gy 5-FU 225 mg/m^2 CI Bevacizum ab (10 mg/kg every 2 weeks)	40	40

CI = continuous; i.v. = infusion; NR = not reported.
[a]Dose Limiting Toxicities observed.
[b]Recommended dose.

m^2 intravenous given 1 week before the beginning of radiation followed by 250 mg/m^2/week for 5 week) and radiation therapy (45 Gy) was 825 mg/m^2 twice-daily continuously during radiotherapy including weekends. In this study, the pathological complete response rate was only 5% (2 of 40 patients) but a significant number of patients experienced T/N tumor downstaging as well as tumor regression according to the criteria of Wheeler and/or Dworak. The safety profile of capecitabine and cetuximab was favorable and treatment related toxicity was manageable in most patients. Diarrhea (15%) was the most frequent grade 3 toxicity and appeared to be slightly more frequent than in other trials of capecitabine and radiotherapy without cetuximab (< 5%). Two grade 4 vascular events were observed: one

pulmonary embolism and one myocardial infarction. Recently, the addition of gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, to capecitabine (650 mg/m^2 twice-daily) and radiotherapy in patients with localised rectal and pancreatic cancer has been associated with significant toxicity in particular diarrhea and arterial thrombosis (Czito *et al*., 2006). Whether epidermal growth factor receptor inhibition in combination with radiotherapy and capecitabine increases the risk of such events is a question that will have to be carefully looked at in future trials.

In a very preliminary report, pathological complete response was observed in 12% of patients treated with a regimen combining 5-fluorouracil, cetuximab and preoperative radiation therapy (Chung *et al*., 2006). Of note, the combination of cetuximab and capecitabine with either irinotecan or oxaliplatin is also under investigation, in association with radiation therapy in the preoperative treatment of patients with locally-advanced rectal cancer (Arnold *et al*., 2006; Hofheinz *et al*., 2006).

Bevacizumab (a vascular endothelial growth factor-targeted antibody) given in combination with chemotherapy improves survival over chemotherapy alone in patients with metastatic colorectal cancer (Hurwitz *et al*., 2004). In the preoperative treatment of rectal cancer, bevacizumab has been combined with 5-fluorouracil (225 mg/m^2/day continuous infusion) and radiotherapy (50.4 Gy). Bevacizumab at the dose of 5 mg/kg every 2 weeks was well tolerated. However, at the second dose level (10 mg/kg every 2 weeks), two of five patients developed dose-limiting toxicities: diarrhea and colitis. Importantly, it has also been shown that a single infusion of bevacizumab induced a significant decrease in tumor microvascular density and normalized tumor vascularisation (Willett *et al*., 2004, 2005).

CONCLUSION

One universal finding in studies comparing chemoradiation to radiotherapy alone or preoperative to postoperative treatment is that with either approach there is no significant impact on systemic recurrence or survival rates, which is probably affected only by the use of appropriate, full dose and sufficiently long systemic chemotherapy. Preoperative administration of either radiotherapy or chemoradiation appears more effective and less toxic than postoperative administration. In the context of suboptimal surgery (i.e., non total mesorectal excision), chemoradiation is possibly superior to preoperative radiotherapy alone in reducing loco-regional failure. In the context of optimal surgery, this assumption may still apply to patients at high risk of loco-regional failure, but the benefit obtained with the addition of chemotherapy to preoperative radiotherapy in low risk patients is currently unknown.

New schedules of chemoradiation with oral fluoropyrimidines appear to be at least as good as and probably more convenient than 5-fluorouracil-based regimens, though there have been no randomized comparisons to date. Promising new schedules with highly active cytotoxic and targeted agents such as oxaliplatin, cetuximab and bevacizumab are being intensively investigated. Although at the present time none of these regimens can be considered standard treatment, results of ongoing and planned randomized clinical trials investigating the role of these compounds in rectal cancer in combination with radiation therapy are eagerly awaited.

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23
Resectable Rectal Cancer: Preoperative Short-Course Radiation

Krzysztof Bujko

INTRODUCTION

Two randomized trials have demonstrated that preoperative radiotherapy is superior to postoperative radiotherapy in its ability to decrease local recurrence for rectal cancer (Frykholm *et al*., 1993; Sauer *et al*., 2004). In addition, the rates of early and late adverse effects have been lower with preoperative setting. The short-course radiation which consists of five fractions of 5 Gy delivered during 5 days with surgery carried out during the next week is the most extensively studied preoperative radiotherapy schedule in the frame of randomized studies. This schedule is commonly used as a routine treatment for resectable rectal cancer in Northern Europe. In contrast, in Southern Europe and in the U.S.A., conventionally fractionated radiotherapy (45–50 Gy, 1.8 or 2 Gy per fraction) given concurrently with chemotherapy and with surgery carried out 4–8 weeks later is preferred. The aim of this chapter is to provide theoretical rationale for the short-course radiation, to present its variants, long-term outcomes, early and late adverse effects and to evaluate the advantages and limitations of its use in relation to preoperative chemoradiation.

SHORT-COURSE SCHEDULE

Although primary rectal cancer grows slowly, which is reflected in a long volume doubling time, its cells proliferate rapidly (Rew *et al*., 1991). The slow tumor growth is caused by large cell loss exceeding 90%. A potential volume doubling time is the measure of proliferation activity. This parameter denotes the period in which a tumor would double its volume assuming that cell loss does not exist. The mean potential doubling time for rectal cancer is as short as 5 days (Rew *et al*., 1991). This observation has led to the concept of shortening the overall irradiation time to limit the ability of cancer cells to repopulate. This concept was supported by the animal experiment reported by Basha *et al*. (2002). In this study, tumors were irradiated with 5 × 5 Gy during 5 days or 10 × 3 Gy during 10 days or 10 × 3 Gy (twice per day) during 5 days. Significantly higher degree of cell killing was observed after both schedules of radiation delivered during 5 days compared to that delivered during 10 days.

As has been demonstrated in the above experiment, the overall treatment time might be shortened by increasing the dose

per fraction (hypofractionation) or by delivering conventional (or similar to conventional) doses per fraction two or three times per day (accelerated regimen). The advantages of hypofractionated regimens over accelerated regimens are convenience (savings in time for patients and departments) and lower costs. These advantages are especially evident in departments with long waiting lists. On the other hand, large dose per fraction leads to the concern of increasing risk of late adverse effects. It should be noted, however, that regarding late adverse effects, biologically effective dose of 25 Gy delivered with 5 Gy per fraction is slightly smaller compared to that calculated for 50 Gy fractionated conventionally (Table 23.1). Despite this calculation, there is still a concern of late adverse affects. For this reason, accelerated regimens using doses per fraction in the range of 1.5–2.5 Gy delivered two or three times per day have been proposed (Table 23.2).

For many years, it was commonly believed that the alpha/beta ratio was high for a tumor control and amounted to approximately 10 Gy. Thus, there was concern that 25 Gy might be too low for tumor control. However, recent data demonstrated that for breast adenocarcinoma alpha/beta estimate is low (5 Gy) and similar to the values calculated for late adverse effects (Dewar *et al*., 2007). Also, for rectal adenocarcinoma the alpha/beta ratio is suggested to be low (5 Gy) (Suwinski *et al*., 2007). Table 23.1 demonstrates that the radiobiological estimates of short-course schedule are similar to that calculated for conventionally fractionated schedule, when the time-corrected linear-quadratic model and this low alpha/beta ratio for tumor control were used.

With the short-course preoperative radiotherapy, surgery is commonly carried out within the next week. Thus, an early toxicity is expected to be low because early adverse effects, most often tenesmus and urgency, occur ~ 1 week after completion of the short-course radiation (Bujko, 2007). Thus, the organ (rectum) at risk for acute toxicity is removed before symptoms of early post irradiation damage occur.

Shortly after irradiation, nonviable cancer cells may look morphologically intact

TABLE 23.1. Comparison of the short-course radiation with the conventionally fractionated radiation base on calculations of biologically effective doses (BED) according to the linear-quadratic model.

	BED for tumor control probability, $\alpha/\beta = 5$ Gy		BED for late normal tissue complication probability, $\alpha/\beta = 3$ Gy
Schedule	Without time correction	With time correction	
25 Gy, 5 Gy per fraction, 5 days	50	50	66.7
50 Gy, 2 Gy per fraction, 33 days	70	54.4	83.3

BED was calculated using the following formula: $BED = nd\,(1 + d/\alpha/\beta)$, where n = number of fraction, d = dose per fraction, α/β = linear-quadratic quotient. BED with time correction was calculated by subtracting from BED a dose lost to counteract tumor cells repopulation assuming that 0.6 Gy counteracts daily tumor repopulation and that repopulation delay is 7 days (Fowler, 1989). Formula: $BED_{corr} = BED - 0.6\,(T - 7)$, where T = overall treatment time. If overall treatment time equals or is less than 7 days, no time correction is required.

TABLE 23.2. The non-randomized trials testing short-course preoperative radiotherapy.

Reference	Study design	Number of patients	Median follow-up (years)	Main findings
Brooks *et al*., 2006	25 Gy given in 1.67 Gy per fraction three times per day over 5 days; surgery within 7 days	20	2.5	Tolerable early toxicity; local control 95% at 3 years
Coucke *et al*., 2006	41.6 Gy given in 1.6 Gy per fraction twice per day over 17 days; surgery within 7 days	279	4.5	Tolerable early toxicity; local control 92% at 5 years
Suwinski *et al*., 2006	42 Gy given in 1.5 Gy per fraction twice per day over 18 days; surgery after 6 days (median)	62	2.9	Tolerable early toxicity; local control 94% at 3 years
Voelter *et al*., 2006	CPT-11 delivered concomitantly with 41.6 Gy given in 1.6 Gy per fraction twice per day over 17 days; surgery within 7 days	33	2	Severe diarrhea in 24% of patients; local control 100% at 4 years
Widder *et al*., 2005a	25 Gy given in 2.5 Gy per fraction twice per day over 5 days; surgery within 7 days	184	3.5	Tolerable early toxicity

(Suit and Gallager, 1964), but a few days or weeks later, those cells undergo necrosis (Marijnen *et al*., 2001). This explains why there is limited tumor shrinkage and no downstaging, if the interval between the onset of short-course radiation and surgery is shorter than 10 days (Marijnen *et al*., 2001). This also explains why, despite similar local efficacy, the rate of complete tumor response is much lower and the rate of positive circumferential margin is much higher after the short-course radiation with immediate surgery than after the chemoradiation with long interval to surgery (Bujko *et al*., 2004, 2006b). The short-course radiotherapy with immediate surgery cannot be used when tumor shrinkage is required, for example, in patients with initially unresectable tumor. The ongoing Stockholm III trial compares 5 × 5 Gy and immediate surgery vs. 5 × 5 Gy and delayed surgery vs. conventionally fractionated 50 Gy and delayed surgery (Glimelius, 2006). This trial will provide an answer to the question of whether long interval between the short-course radiation and surgery yields similar downsizing and downstaging effect to that seen after conventional fractionation. In conclusion, from theoretical perspective the main benefit of short-course schedule is a reduction of overall treatment time, low early toxicity, convenience, and low costs.

TESTING THE SHORT-COURSE PREOPERATIVE RADIOTHERAPY: RANDOMIZED TRIALS

Systematic literature search was carried out without language restrictions in order to identify randomized trials testing the short-course preoperative radiotherapy.

The PubMed and Cochrane databases were searched through August 2007 applying the following keywords: (rectal cancer or rectal adenocarcinoma) and (randomized or randomised or preoperative radiotherapy). The computerized search was supplemented with hand searches of reference lists of all available review articles, original studies, bibliographies of books, and abstracts from ASTRO, ESTRO and ASCO meetings (2002–2007). The studies were eligible if they met the following criteria: (i) randomized clinical trial for rectal adenocarcinoma; (ii) evaluation of short-course preoperative radiotherapy defined as carried out within the time shorter than 2 weeks; (iii) available data regarding survival and local control. The results of this literature search are presented in Table 23.3. Twelve trials were identified. All of these trials in experimental arms used 5 Gy per fraction and the surgery was carried out within the subsequent week.

Local Efficacy and Survival

Seven trials have compared preoperative radiotherapy and immediate surgery with surgery alone (Table 23.3). Two of those trials, which compared one fraction of 5 Gy followed by surgery with surgery alone, have not shown the benefit in local control and survival (Rider *et al*., 1977; MRC Working Party, 1984). By using three fractions of 5 Gy prior to surgery, a small reduction of the risk of local recurrence was reported; from 24% with surgery alone to 17% with the addition of radiotherapy (29% of relative reduction) (Goldberg *et al*., 1994). By using four fractions of 5 Gy (Marsh *et al*., 1994) or five fractions of 5 Gy (Stockholm Colorectal Cancer Study Group, 1990; Martling *et al*., 2001; Folkesson *et al*., 2005) the relative reduction of local recurrences was higher and amounted to 65% and 52–65%, respectively. Among the four last trials, in the largest one (1,168 patients) 8% benefit in overall survival at 13 years was reported (Folkesson *et al*., 2005). In the remaining trials, benefit in disease-specific survival or in overall survival in the subgroup of curatively treated patients was demonstrated (Marsh *et al*., 1994; Stockholm Colorectal Cancer Study Group, 1990; Martling *et al*., 2001).

One trial compared preoperative radiotherapy (5 × 5.1 Gy) with conventionally fractionated postoperative radiotherapy (60 Gy, 2 Gy per fraction) (Frykholm *et al*., 1993). Superior local control and lower rate of late complications were reported in the preoperative radiotherapy group. Survival did not differ between the groups. All aforementioned trials were conducted before the era of modern surgery technique, total mesorectal excision (TME). Thus, local recurrences rates were higher than presently reported.

Two other large trials, TME trial (Marijnen *et al*., 2005) and MRC CR07 trial (Sebag-Montfiore *et al*., 2006) have compared preoperative radiotherapy and immediate surgery with selective use of postoperative radiochemotherapy for patients at high risk for local recurrence, namely for those with positive circumferential resection margin. Both trials aimed to answer the question of whether preoperative radiotherapy is still needed after TME, bearing in mind a low local recurrence rate reported after this technique. The second aim was to find out whether preoperative radiation was justifiable for routine use, which meant putting many patients at risk for adverse effects, or should the

TABLE 23.3. The randomized trials testing short-course preoperative radiotherapy.

Study name, reference	Study design	Number of patients	Median follow-up (years)	Main findings
Toronto trial, Rider *et al*., 1977	1 × 5 Gy preoperatively vs. surgery alone	125	5	No differences in survival; local control rate not given
MRC 1, MRC Working Party, 1984	1 × 5 Gy preoperatively vs. 20 Gy, 2 Gy per fraction preoperatively vs. surgery alone	824	5	No differences in survival, local control and postoperative complications.
Stockholm 1, Stockholm Colorectal Cancer Study Group, 1990	5 × 5 Gy with immediate surgery vs. surgery alone	849	4.5	No differences in overall survival. Radiotherapy better disease specific survival, p = .05, less local recurrences (11% vs. 23%, p < .01) higher postoperative mortality (8% vs. 2%, p < .01).
Uppsala, Pahlman *et al*., 1985; Frykholm *et al*., 1993	5 × 5.1 Gy with immediate surgery vs. postoperative radiotherapy 60 Gy, 2 Gy per fraction	471	Minimum 5	No differences in overall survival, p = .5. With preparative radiotherapy less local recurrences (13% vs. 22%, p = .02) and late small bowel obstruction (5% vs. 11%, p < .01)
St Mark's hospital, Goldberg *et al*., 1994	3 × 5 Gy with immediate surgery vs. surgery alone	468	Minimum 5	No difference in overall survival, p = .92). With radiotherapy less local recurrences (17% vs. 24%, p = .04) and higher postoperative mortality (9% vs. 4%, p < .05).
NW England, Marsh *et al*., 1994	4 × 5 Gy with immediate surgery vs. surgery alone	284	Minimum 8	Similar overall and cancer related mortality (p = .21 and .09). With radiotherapy less local recurrences (13% vs. 37% p < .001) and better survival for curatively treated patients, p = .033.
Stockholm 2, Martling *et al*., 2001	5 × 5 Gy with immediate surgery vs. surgery alone	557	9	No difference in overall survival for all patients. With radiotherapy better overall survival for curatively treated patients (46% vs. 39%, p = .03) less local recurrences (12% vs. 25%, p < .001) and more deaths of intercurrence disease within 6 months of surgery (5% vs. 1%, p = .02)
Swedish trial, Swedish Rectal Cancer Trial, 1993 and 1997 Cancer Trial, 1993 Dahlberg *et al*., 1998 Folkesson *et al*., 2005 Birgisson *et al*., 2005	5 × 5 Gy with immediate surgery vs. surgery alone	1,168	13	With radiotherapy better overall survival (38% vs. 30%, p = .008) and less local recurrences (9% vs. 26%, p < .001) at 13 years.

(continued)

TABLE 23.3. The randomized trials testing short-course preoperative radiotherapy.

Study name, reference	Study design	Number of patients	Median follow-up (years)	Main findings
TME trial, Kapiteijn *et al*., 2002 Marijnen *et al*., 2001 Marijnen *et al*., 2002 Marijnen *et al*., 2004 Marijnen *et al*., 2005 Peeters *et al*., 2005	5 × 5 Gy with immediate surgery vs. surgery alone or postoperative radiotherapy (50.4 Gy, 1.8 Gy per fraction) for patients with positive circumferential resection margin.	1,861	5	At 5 years no difference in overall survival. With radiotherapy less local recurrences (6% vs. 11%, $p < .001$).
MRC CR07, Sebag-Montfiore *et al*., 2006	5 × 5 Gy with immediate surgery vs. surgery alone or postoperative chemoradiation for patients with positive circumferential resection margin	1,350	3	At 3 years no difference in overall survival. With radiotherapy statistically significant better disease-free survival (80% vs. 75%) and less local recurrences (5% vs. 11%).
Polish trial, Bujko *et al*., 2004 Bujko *et al*., 2006b; Pietrzak *et al*., 2007	5 × 5 Gy with immediate surgery vs. chemoradiation (50.4 Gy, 1.8 Gy per fraction + 5-Fu, LV) with delayed surgery	312	4	No difference in sphincter preservation (main end-point). At 4 years no differences in survival, disease-free survival, local recurrences and late toxicity.
TROG 0104 trial, Ngan *et al*., 2007	5 × 5 Gy with immediate surgery vs. chemoradiation (50.4 Gy, 1.8 Gy per fraction + 5-Fu, LV)	326	–	With 5 × 5 Gy less postradiation acute toxicity, 1.9% vs. 28%, $p < .001$; similar rate of postoperative complications

indication for radiotherapy be limited for those with the highest risk of local recurrence? Both trials have demonstrated significant benefit of radiotherapy in terms of local control. The local recurrence rate in the preoperative radiotherapy group as compared to the surgery-alone group was 6% vs. 11% at 5 years in the TME trial and 5% vs. 11% at 3 years in the MRC CR07 trial, respectively. The relative efficacy of 5 × 5 Gy in reducing the rate of local recurrence was similar to that seen in the pre-TME era and amounted to ~ 50%. Although in terms of absolute percentages, this benefit is smaller compared to that seen in pre-TME era; 5%–6% vs. 12%–17%. Both trials did not demonstrate benefit in overall survival. In the MRC CR07 trial disease-free survival was significantly better in preoperatively irradiated group, 80% vs. 75%. This, however, was reported as actuarial figure at 3 years with many patients having follow-up time of < 1 year. Thus, the data is not mature enough for final assessment. In the TME trial there was no difference in disease-free survival.

Early Adverse Effects and Compliance

As mentioned earlier, when surgery is carried out within a week after radiation, then the main organ (rectum) at risk for early toxicity is removed before adverse effects occur. The symptoms of radiation acute toxicity, such as enteritis, cystitis, and dermatitis may occur with a delay of approximately 1 or 2 weeks after irradiation; therefore, within an early postoperative period. For this reason, an early toxicity of short-course schedule is probably underreported, as it is hardly distinguished from postoperative complications. Indeed, the rate of early toxicity after short-course schedule has been reported to be much lower than after conventionally fractionated radiation. In the Uppsala trial (Pahlman *et al*., 1985) that had compared the short-course preoperative radiotherapy with the conventionally fractionated postoperative radiotherapy (Table 23.1), adherence to the short-course radiation schedule was 99% and no acute toxicity was reported; whereas, in the postoperative radiation group, acute toxicity was observed in 92% of patients. In addition, because of prolonged recovery after surgery, 6% of patients did not receive postoperative radiotherapy and in 21% of patients, the interval between surgery and radiotherapy was delayed for > 8 weeks. The other trials confirmed excellent compliance (> 95% of patients) to the 5 × 5 Gy schedule (Kapiteijn *et al*., 2001; Martling *et al*., 2001; Swedish Rectal Cancer Trial, 1993).

The detailed data about acute toxicity of 5 × 5 Gy was presented by Marijnen *et al*. (2002) based on the TME trial. Any early adverse effects were reported in 26% of patients and in 7% of them, the complications were recorded as severe (grade 2 or 3). Gastrointestinal side effects were more frequently observed. Sacral pain, usually of short duration, was reported in 10% of patients. Majority of those patients did not require any intervention; in 2.5% of patients, the pain was severe and required treatment interruption.

Postoperative Complications

In the Stockholm 1 trial (Stockholm Colorectal Cancer Study Group, 1990) and in the St Mark's Hospital trial (Goldberg *et al*., 1994) an increased postoperative mortality was recorded in

patients given radiotherapy compared with unirradiated patients (8% vs. 2% and 9% vs. 4%, respectively). Combined analysis of Stockholm 1, Stockholm 2 and Uppsala trial revealed that the increase of mortality was related to a two-portal radiotherapy technique to a relatively large volume and was not observed in patients treated with a four-portal technique to a limited volume (Holm *et al*., 1996). In trials in which modern radiotherapy technique was used, there was no difference in postoperative mortality between radiotherapy plus surgery group compared to surgery-alone group (Swedish Rectal Cancer Trial, 1993; Kapiteijn *et al*., 2001).

Based on the material from the TME trial, detailed information regarding postoperative complications with the use of modern radiotherapy and surgical techniques were reported by Marijnen *et al*. (2002). There was no difference in median operation time and hospital stay between the 5 × 5 Gy preoperative radiotherapy group and the surgery-alone group. The blood loss was slightly increased in the radiotherapy group (100 mL). The overall postoperative complications rate was higher in the radiotherapy group, 48% vs. 41%, p = .008. This difference was mainly attributed to the variations in the perineal wound complications, 29% vs. 18%, p = .008. Among patients receiving irradiation, the perineal wound healing problems were more frequently observed in those in whom the perineum was included in the treatment volume. The rate of anastomotic leakage did not differ between groups. The above is in concordance with other reports of randomized trials (Goldberg *et al*., 1994; Swedish Rectal Cancer Trial, 1993; Pahlman *et al*., 1985; Stockholm Rectal Cancer Study Group, 1990).

Late Adverse Effects

Based on the Swedish Rectal Cancer Trial, Birgisson *et al*. (2005) provided accurate data regarding late adverse effects after short-course radiotherapy. Minimal follow-up time was 11 years. The data were retrieved from the register that included all hospital admissions. No difference in the risk for overall admission to the hospital was found between the irradiated group and surgery-alone group (relative risk (RR) = 1.07, 95% confidence interval (CI) 0.91–1.26). However, an increased risk for admission was seen in irradiated patients during the first 6 months after treatment (RR = 1.64, 95% CI 1.21–2.22). The main reasons for this increased risk were gastrointestinal disorders and infections. Eight years after treatment, a slight increase of admissions to the hospital for bowel obstruction, nausea, and unspecific abdominal pain was recorded. A trend for higher rate of bowel obstructions was noted for patients irradiated with two-field technique as compared to those treated with multiple fields.

The results presented above rule out considerable increased risk of severe late adverse effects after radiation of such importance as to result in hospital admission. This, however, did not indicate late toxicity not requiring hospitalization but potentially impairing patients' quality of life. Peeters *et al*. (2005) and Marijnen *et al*. (2005), provided data regarding sexual and bowel functions and quality of life after short-course preoperative radiotherapy in comparison with surgery alone based on large Dutch TME trial. Increased rates of fecal incontinence (62% vs. 38%,

P < .001), stool frequency for patients without stoma (median 3.69 vs. 3.02, p = .011), sexual disorders in males (p = .004) and females (p < .001) were reported in the irradiated group compared to the surgery alone group. In the irradiated patients, bowel dysfunction had a greater negative impact on daily activities than in the surgery-alone group, 34% vs. 22%, p = .01. Despite an increase in bowel and sexual dysfunctions in irradiated groups, the formally measured health-related quality of life was similar in irradiated and surgery-only groups (Marijnen *et al*., 2005). Those results may be explained by the low sensitivity of the formal tools used to measure quality of life in the detection of differences in the treatment toxicity.

The concern of the 5 × 5 Gy preoperative radiotherapy is the late chronic neurotoxicity. During the follow-up period which ranged from 3 to 14 years, generally reversible sacral pain of long duration was reported in 7 of 503 patients (1.4%) and 3 (0.6%) of them also developed other neurological symptoms such as weakness, numbness, and parenthesis of lower extremities (Frykholm *et al*., 1996). This neurotoxic effect, however, did not translate to the detectable difference between answers to the questionnaire regarding neurologic functions in the 5 × 5 Gy group and the surgery-alone group of Dutch TME trial (Peeters *et al*., 2005). Similarly, in the Swedish trial there was no difference between both groups in the hospital admissions due to the neurological disorders (Birgisson *et al*., 2005).

In the combined material of Stockholm I and II trials, 5.3% of irradiated patients and 2%, 4% of non-irradiated patients (p = .03) were hospitalized because of femoral or pelvic fractures (Holm *et al*., 1996). It might be relevant to point out that in both trials the posterior part of the sacrum was not shielded. No increase in femoral or pelvic fractures after 5 × 5 Gy was observed in the Dutch, Swedish, and Polish trials (Peeters *et al*., 2005; Birgisson *et al*., 2005; Bujko *et al*., 2006b).

TESTING THE SHORT-COURSE PREOPERATIVE RADIOTHERAPY: NON-RANDOMIZED TRIALS

There are numerous publications showing results of phase I–II studies testing a variety of short-course preoperative radiotherapy schedules (Table 23.2). Those schedules, however, have not been compared with routinely used schedules in randomized trials. The majority of those studies used two or three fraction per day in order to keep the overall irradiation time short and to reduce the risk of late complications. Although some of the results of those studies are encouraging, by the nature of their design, the firm conclusions from their findings cannot be drawn with regard to indications for applicability of those schedules. This is due to confounding factors such as case mix and differences in quality of surgery.

ADVANTAGES AND LIMITATIONS OF SHORT-COURSE SCHEDULE

As mentioned in the Introduction, there are two most commonly used schedules of preoperative radiation for resectable rectal cancer: five fractions of 5 Gy delivered

during 5 days with surgery carried out during the next week and chemoradiation consisting of 45–50 Gy delivered in 1.8 or 2 Gy per fraction during 5 weeks concomitantly with chemotherapy and with surgery carried out 4–8 weeks later. Those two schedules were compared directly in two randomized trials: the Polish trial (Bujko *et al*., 2006b) and the TROG 0104 trial (Ngan *et al*., 2007). The TROG trial was closed to accrual recently after 326 patients had been included, thus only acute adverse effects were reported.

The Polish trial, based on 312 randomized patients, aimed at answering the question of whether downsizing effect of preoperative chemoradiation results in an improved rate of sphincters preservation when compared to preoperative short-course radiotherapy. The anterior resection rate did not differ in the both groups and amounted to 61% in the short-course group and 58% in the chemoradiation group, p = .57. The overall early radiation toxicity was higher in the chemoradiation group; 85% vs. 25%, p < .001. The corresponding figures for grade 3 or 4 acute toxicity were 3% vs. 18%, p < .001 in the Polish trial and 1.9% vs. 28%, p < .001 in the TROG trial. This lower toxicity of short-course radiation translated to a better compliance to short-course radiation protocol than to chemoradiation protocol; 98% vs. 69%. In the Polish study, two toxic deaths (1.5%) were reported in the chemoradiation arm. It should be stressed that no deaths due to the acute toxicity have been reported in patients treated with 5 × 5 Gy preoperative radiotherapy (Kapiteijn *et al*., 2001; Martling *et al*., 2001; Swedish Rectal Cancer Trial, 1993; Pahlman *et al*., 1985); whereas, with preoperative radiochemotherapy mortality due to the acute toxicity varied between 0.4% and 1.7% (Hyams *et al*., 1997; Bosset *et al*., 2004; Bujko *et al*., 2004). The incidence of postoperative complications after the short-course radiation was similar to that observed after chemoradiation. In the Polish trial 23% of patients in the short-course group had complications and 15% of patients in the chemoradiation group, p = .27 (Bujko *et al*., 2004). The corresponding figures in the TROG trial were 51% vs. 49% (Ngan *et al*., 2007). In the Polish trial, there was no significant difference in survival and local control between both groups. The actuarial 4-year (median follow-up) overall survival was 67% in the short-course group and 66% in the chemoradiation group, p = .96. The corresponding values for disease-free survival were 58% vs. 56%, p = .82 and for crude incidence of local recurrence 9% vs. 14%, p = .17. The limitations of survival and local control analysis should be acknowledged. The study was underpowered to detect a small difference in outcomes, as it has been designed to detect differences of 15% or greater in sphincter preservation. No increase of late toxicity in the short-course irradiation arm was found, although admittedly, the follow-up was too short (median 4 years) to draw any definitive conclusions. The crude overall incidence of late toxicity was 28% for patients in the short-course group and 27% in the chemoradiation group, p = .81 (Bujko *et al*., 2006b). The corresponding values of severe late toxicity were 10% vs. 7%, p = .36. There was no difference in late occurring neurological disorders between 5 × 5 Gy arm and chemoradiation arm. No significant differences were observed between the randomized groups regarding quality of life, the anorectal and sexual functions (Pietrzak *et al*., 2007).

Due to the uncertainty in to toxicity, which may result from an interaction between

drugs and radiation delivered with high doses per fraction, simultaneous use of short-course radiation with neither chemotherapy nor biologicals, such as cetuximab or bevacizumab, has been tested. It is unknown whether with the development of more effective radiosenitizing agents, 5 × 5 Gy schedule would still be an alternative to conventional or accelerated fractionation. The sequential use of 5 × 5 Gy and chemotherapy seems to be feasible and effective. Widde *et al*. (2005b) reported on two patients who received 5 × 5 Gy followed by three courses of oxaliplatin and capecitabine prior to surgery. Symptoms decreased rapidly after irradiation. Early tolerance was acceptable. In both cases, complete pathological response in postoperative specimen was reported.

In conclusion, the long-term outcomes after short-course radiation were similar to that observed after chemoradiation. Tumor shrinkage induced by preoperative chemoradiation did not translate in the higher rate of anterior resection. The result of metaanalysis of other trials is in concordance with this finding (Bujko *et al*., 2006a). This suggests that preoperative 5 × 5 Gy with immediate surgery and preoperative conventionally fractionated chemoradiation with delayed surgery might be considered as alternative options for patients with resectable lesions. Based on the results of the Polish and the TROG studies and trials presented in Table 23.3, the 5 × 5 Gy schedule is being used in Poland preoperatively for resectable rectal cancer due to its lower early toxicity, better compliance, and lower cost, as compared with preoperative chemoradiation.

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24
Preoperative Chemoradiotherapy Allows for Local Control in Rectal Cancer – But Distant Metastases Remain an Unsolved Problem

Verena Voelter

INTRODUCTION

Colorectal cancer represents a major health care problem because it is the second leading cause of cancer related deaths in Europe and the third cause in the U.S. (Jemal *et al*., 2007). Adenocarcinoma of the colon and the rectum is commonly referred to as a single disease, although rectal carcinoma is a distinct entity, with particular biologic and genetic features and clinical behavior. Whereas local recurrence is rare in colon cancer, it is a common event in rectal cancer. The particular anatomical location of the rectum within the narrow margins of the pelvis without a peritoneal cover renders local spread a common event with tumor deposits occurring in the perirectal fat as well as infiltration of the locoregional lymph nodes.

During the last 2 decades many landmark trials have added important information to the field and helped to improve the outcome of these patients. Surgery has remained the cornerstone in the multimodal treatment of rectal cancer, and the progress has been particularly evident for the surgical procedure with the introduction of total mesorectal excision. The introduction of complementary treatments such as irradiation and chemotherapy allowed for an additional considerable reduction of local recurrence rates. The trimodality approach comprising surgery, radiotherapy, and chemotherapy has now become standard for locally advanced rectal cancer patients. However, the overall mortality for locally advanced rectal cancer patients remains unchanged with an incidence of ~ 40% at 5 years due to the occurrence of distant metastases representing a major unsolved problem (Bosset *et al*., 2006; Greene *et al*., 2004; Marijnen *et al*., 2005; Sauer *et al*., 2004).

In addition to 5-fluorouracil, new potent compounds such as irinotecan and oxaliplatin became available displaying increased therapeutic activity. In the era of modern targeted therapies, the so-called biological agents, e.g., the monoclonal antibodies cetuximab and bevacizumab, further enrich the arsenal of antitumor drugs, and they consistently augment the activity of standard chemotherapy. Overall, the median survival of patients with metastatic colorectal cancer has more than doubled

from 8 months to over 20 months since the introduction of these new compounds. It is likely that these new agents account for the observed decline in colorectal cancer related mortality during the last 2 decades (Jemal *et al*., 2007).

Here, the chronological introduction of each of the three therapeutic cornerstones will be discussed as well as new chemotherapeutic and biological agents that are currently under investigation. The introduction of the new antitumor agents may harbor the potential to improve on overall patient's outcome in the future.

SURGERY

The local recurrence rate after conventional rectal surgery is high and the disease recurs within the pelvis in almost one out of three patients operated (MacFarlane *et al*., 1993). The total mesorectal dissection was developed 30 years ago by Heald (1998). He demonstrated that a sharp dissection of the mesorectum along the narrow pelvic structures is feasible without increased toxicity leading to prevention of local recurrence in the vast majority of patients. Indeed, since the introduction of total mesorectal dissection in the 1990s, local recurrence rates have dramatically dropped from ~ 40% under the 10% threshold and very experienced surgeons in high volume centers now even accomplish a local failure rate of below 5% (Heald *et al*., 1998).

The Dutch trial that investigated preoperative radiotherapy before total mesorectal dissection was carried out nationwide in high-volume community hospitals as well as in academic institutions (Kapiteijn *et al*., 2001). It had incorporated a detailed training program for all surgeons participating in the trial. They demonstrated a considerable decline in abdomino-perineal resection rate (resection of the anal sphincter), which can be considered as a surrogate for the efficacy of the preoperative treatment due to the introduction of total mesorectal dissection and a successful training program. This trial is elusive for another reason: a local recurrence rate of 11% with total mesorectal dissection alone probably reflects better the real life outside the very specialized academic centers that report local recurrence rates below 5% claiming that no additional treatment is necessary for these patients (Heald *et al*., 1998; Marijnen *et al*., 2005). In conclusion, total mesorectal dissection is now recognized as the standard surgical procedure for rectal cancer patients worldwide.

PREOPERATIVE VERSUS POSTOPERATIVE RADIOTHERAPY

Postoperative radiotherapy has been added to conventional surgery in the early rectal cancer trials since the 1980s. It allowed for a 50% reduction in local recurrence rate and was then included as a second cornerstone in the treatment of locally advanced rectal carcinoma (GTSG, 1985). However, the timing of radiotherapy, before or after surgery, has been controversial for many years, and a commonly accepted standard has only evolved in the beginning of the third millennium. One single randomized trial compared preoperative versus postoperative short-term radiotherapy (5×5 Gy), and the results published in the early 1990s were striking; local failure was significantly reduced by half with the preoperative treatment. However, overall survival was

identical with ~ 50% of patients alive at 5 years (Pahlman and Glimelius, 1990). This was confirmed later by two metaanalyses demonstrating an advantage in favor of preoperative radiotherapy (Camma *et al*., 2000; CRC, 2001).

Furthermore, the randomized trial confirmed the prior nonrandomized experience that preoperative radiotherapy is not correlated with increased toxicity and does not compromise the following surgery as suspected by many radio/oncologists at that time (Pahlman and Glimelius, 1990). It was proven to be even less toxic than the postoperative approach and, more importantly, it became evident that half of the patients were not able to start radiotherapy in time after surgery due to prolonged postoperative recovery. In contrast, almost all patients of the preoperative group received the whole treatment modality of radiotherapy and surgery.

The European community started investigating the role of preoperative radiotherapy in the 1980s and in the beginning several studies using conventional long-term radiotherapy did not establish an advantage over conventional surgery alone. This was in part due to high toxicity that has been substantially improved with modern radiation techniques. In the mid-1980s, the Swedish Rectal Cancer Trial (SRCT) compared short-term preoperative radiotherapy (5 × 5 Gy) to conventional surgery alone and demonstrated for the first time a significant increase in overall survival in addition to reduced local recurrence in patients treated with preoperative irradiation (SRCT, 1997). The impact on survival was then confirmed by the two metaanalyses (Camma *et al*., 2000; CRC, 2001).

The maintained value of radiotherapy in the era of total mesorectal excision has been underlined in the large multicenter Dutch trial showing a significant benefit for short-term radiotherapy delivered 1 week before total mesorectal excision (Kapiteijn *et al*., 2001). The results underline that preoperative radiotherapy cannot be omitted in this patient population at high risk of local relapse with a reduction of local failure by half from 11% to 6%.

Nevertheless, the hypofractionated schedule using 5 Gy per day during 5 consecutive days is associated with considerable local toxicity. Radiotherapy induced toxicity is on the one hand correlated with the dose delivered per fraction, and on the other hand with the total cumulative dose. In order to reduce toxicity while keeping a dose-dense schedule and immediate surgery after 1 week, as performed in the SRCT and the Dutch trials (Kapiteijn *et al*., 2001; SRCT, 1997), the hyperfractionated regimen HART has been developed. HART uses Hyperfractionated Accelerated RadioTherapy with 1.8 Gy per fraction twice daily. We demonstrated that this regimen is very efficient in yielding high local control rates of > 90% with reduced toxicity compared to hypofractionated schedules used in the SRCT (Coucke *et al*., 2006).

Based on these results, preoperative radiotherapy using hypo- or hyperfractionated schedules evolved to a widely applied treatment option in the beginning of the 2000s. However, one important question remained unsolved: what is the role of chemotherapy combined with preoperative radiotherapy? Indeed, the high incidence of distant metastases and the experience from the postoperative setting with combined chemoradiotherapy leading to prolonged survival urged the investigation of chemotherapy from the overall treatment start.

RATIONALE FOR COMBINED CHEMORADIOTHERAPY

The paradigm of adding systemic chemotherapy to radiotherapy is based on several hypotheses that have already been developed 3 decades ago. The concepts of 'spatial cooperation' of chemotherapy and radiotherapy, as well as 'toxicity independence', 'enhancement of tumor response' and 'protection of normal tissue' were first described in the late 1970s (Steel and Peckham, 1979).

In the meantime, an increasing amount of evidence became available from preclinical *in vitro* and *in vivo* experiments confirming that the concomitant administration of cytotoxic agents and radiotherapy improves the overall therapeutic efficacy. The paradigm of chemoradiotherapy has now been well established in the clinics. In a variety of human cancers it is applied preoperatively, such as for esophageal or, rectal cancer, or as definite treatment, e.g., for anal cancer and locally advanced cervical cancer. The main clinical goals are improvement of local control and avoiding tumor recurrence as well as organ preservation (e.g., larynx, anal sphincter).

From the radiobiological point of view the rationales for combining chemotherapy and radiotherapy are several fold. First, chemotherapy displays an inherent antitumor efficacy when applied with radiotherapy and thus acts synergistically (additive) on tumor cell damage ('spatial cooperation' and 'toxicity independence'). Second, cytotoxic agents that are present during the time interval between irradiation fractions counteract tumor cell repopulation and cellular repair mechanisms, which constitute important elements of radioresistance. In addition, it is admitted that chemotherapeutic agents enhance cytotoxicity against hypoxic tumor cells and lead to cell-cycle synchronization, which in turn increases irradiation damage. Third, chemoradiotherapy improves the radiation dose: tumor cell damage relationship towards an increased induction of cellular growth arrest. In parallel, this radiosensitizing effect of chemotherapy limits radiation-induced toxicity on normal tissue because of a lower radiation dose that can be administered. Ideally, drugs with radiosensitizing properties display supra-additive effects when combined with radiation rather then solely additive, and certainly, infra-additive effects have to be excluded. Lastly, chemotherapy may target systemic micrometastatic disease and thus ameliorate overall disease control in addition to radiotherapy-based local control.

Several chemotherapeutic agents have been employed in chemoradiotherapy: the fluoropyrimidine 5-fluorouracil is one of the most commonly used drugs for rectal and esophageal cancer, and cisplatinum is part of the chemoradiation regimens in head and neck or cervical cancer. Several of the new chemotherapeutic compounds have also been found to display potent radiosensitizing activity like the topoisomerase-I inhibitors of the camptothecin family. Topoisomerase-I (Topo-I) is an enzyme that is involved in DNA synthesis and DNA repair. It functions in the cell during transcription and replication through a process that unwinds supercoiled DNA. Higher levels of Topo-I have been found in malignant cells when compared to healthy tissue. However, the precise mechanisms of radiosensitizing effects of campthotecins are not yet com-

pletely defined. It has been suggested that the reversible cleavable complex formed by the drug and the enzyme ('drug-trapped Topo-I complex') leads to DNA single strand breaks and eventually to G_2-phase cell-cycle arrest. This 'potentially sublethal' DNA damage may be converted to a 'sublethal' and ultimatively to a definite 'lethal' DNA damage cell death with the addition of ionizing radiation (Chen *et al*., 1997).

The timing of the concomitant administration of Topo-I-inhibitors and irradiation seems to be critical: the *in vitro* experiments performed in human tumor cell lines suggest that CPT-11 should be administered before irradiation in order to yield enhanced cytotoxic effects on tumor growth (Chen *et al*., 1997). The preclinical observation of a synergistic activity of Topo-I inhibitors with ionizing radiation led to the hypothesis of an important role of Topo-I in DNA repair during the intervals of radiotherapy fractions. Based on these findings, the campthotecin (CPT-11) has been introduced in clinical chemoradiotherapy regimens for rectal cancer, ensuring the administration of CPT-11 prior to radiotherapy.

CHEMOTHERAPY-RADIOTHERAPY IN THE CLINIC

In the 1980s, postoperative radiotherapy was explored to improve local control in patients at high-risk of pelvic recurrence. However, radiotherapy after surgery did not allow for prolonged survival. Only the addition of the chemotherapeutic agent 5-fluorouracil to the radiotherapy regimen translated in a significant 10% absolute improvement in survival from 50% to 60% at 5 years. In 1990, two well-conducted randomized studies led the NIH to adopt postoperative radiotherapy combined with 5-fluorouracil as new recommendation for clinical stage T3 and T4 or N+ tumors (GTSG, 1985; Krook *et al*., 1991). This subsequently remained the therapeutic standard for more than a decade in the U.S.

In the year 2000, there were two equivalent standard treatment modalities that allowed for better local control *and* improved survival compared to conventional surgery alone: (1) the postoperative combined chemoradiotherapy using 5-fluorouracil that was primarily used in North America and (2) the short-term preoperative irradiation schedule according to the Swedish Rectal Cancer Trial that was widely applied in Europe.

The European Organisation for Research and Treatment of Cancer (EORTC) had already adapted the preoperative irradiation as standard in the EORTC 22921 trial. The trial questioned the value of concomitant chemotherapy with preoperative radiotherapy as well as the role of adjuvant (postoperative) chemotherapy using bolus 5-fluorouracil. Recently, the final results of this large randomized phase III study of > 1,000 patients were reported with a median follow-up of 5.4 years (Bosset *et al*., 2006). The data strengthen the importance of chemotherapy in the multimodality treatment of locally advanced rectal cancer with 17% of the patients who did not receive any chemotherapy experiencing local recurrence compared to 8–10% of patients who were given either preoperative concomitant chemoradiotherapy or postoperative, adjuvant chemotherapy ($p = 0.002$). The overall survival was not different between the groups

(65% at 5 years). Another randomized phase III trial that was conducted in over 700 patients in France recently confirmed these data of preoperative chemoradiotherapy improving local control in the same magnitude (Gerard *et al*., 2006), however, again without prolonging survival (Table 24.1).

There are several explanations for the lack of survival impact of these two important studies that were conceived in the 1980s. First, the surgery applied in the beginning was not total mesorectal excision for the majority of patients (63% in the EORTC trial), which is potentially responsible for the relatively high local recurrence rates. Second, the chemotherapy used was the best available at that time, but certainly 5-fluorouracil does not display sufficient antitumor efficacy. Additionally, the bolus administration of 5-fluorouracil has been demonstrated to be inferior to the continuous infusional regimen that is used in modern chemotherapy schedules. Lastly, the adherence to postoperative chemotherapy in the EORTC trial was very poor with less than half of the patients receiving the treatment, compared to 82% in the preoperative chemotherapy arms. This finding reflects once again the

TABLE 24.1. Randomized phase III landmark trials in rectal cancer.

Established regimen	Author	Treatment arms	Local recurrence (5 years)		Overall survival (5 years)	
Postop CRT	(Krook *et al*., 1991)	SX – RT	25%		~49%	
		SX – CRT	13.5%	p = 0.036	~58%	p = 0.025
Preop short-term RT	(SRCT, 1997)	Ø – SX	27%		48%	
		RT – SX	11%	p < 0.001	58%	p = 0.004
Preop short-term RT before TME	(Marijnen *et al*., 2005)	Ø – SX	11% (5 years)		64%	n.s.
		RT – SX	6%	p < 0.001		
	(Kapiteijn *et al*., 2001)		8.2% (2 years)			
			2.4%			
Preop CRT	(Sauer *et al*., 2004)	SX – CRT	13%		~75%	n.s.
		CRT – SX	6%	p = 0.006		
Preop CRT and adjuvant CT	(Bosset *et al*., 2006)	RT – SX	17%		65%	n.s.
		RT – SX-CT	~9%	p = 0.002		
		CRT – SX				
		CRT – SX-CT				
Preop CRT	(Gerard *et al*., 2006)	RT – SX	16.5%		~68%	n.s.
		CRT – SX	8%	p < 0.05		

Significant numbers are highlighted in red.

Postop, postoperative; Preop, preoperative; SX, surgery; RT, radiotherapy; CT, chemotherapy (all using 5-fluorouracil); CRT, chemoradiotherapy; SRCT, Swedish Rectal Cancer Trial; TME, total mesorectal excision; n.s., not significant.

difficulty of administering radiotherapy or chemoradiotherapy in the postoperative situation with more than half of the patients not benefiting from these additional treatments. However, the use of new potent chemotherapies and targeted therapies during preoperative radiotherapy may allow for an improvement in survival in the future.

PREOPERATIVE VERSUS POSTOPERATIVE CHEMORADIOTHERAPY

Despite the absence of randomized data demonstrating a survival advantage of preoperative chemoradiotherapy over preoperative radiotherapy alone there is strong evidence that the additional administration of systemic antitumor therapy might eradicate micrometastatic disease. One of the major drawbacks of the early trials was that by today's standard suboptimal chemotherapy was employed (5-fluorouracil). More potent compounds are available today and it is likely that the ongoing chemoradiotherapy trials will have an impact on overall outcome of the patients in the future. Furthermore, the rationale of combined preoperative chemoradiotherapy has been extrapolated from the experience in the postoperative situation where it led to significantly prolonged survival.

A number of randomized phase III trials were launched in the late 1990s randomizing preoperative and postoperative chemoradiotherapy, all using 5-fluorouracil (Table 24.1). However, most of these trials were discontinued prematurely because of insufficient accrual (RTOG 94-01 and NSABP R-03). Apparently, investigators encountered difficulties to randomize patients in these trials as the preoperative regimen had grown more popular. The German CAO/ARO/AIO-94 trial has been the only trial that completed accrual and the results are very important to the field (Sauer *et al*., 2004). Local control was shown to be significantly better when preoperative chemoradiotherapy was applied with a 6% versus a 13% incidence of local failure at 5 years ($p = 0.006$). Once again, toxicity was similar between both groups, but the possibility of sphincter sparing surgery seemed to be improved with the preoperative approach.

Based on these landmark trials a change in paradigm occurred in the beginning of this century with the preoperative chemoradiotherapy before total mesorectal excision being now accepted as new therapeutic standard worldwide. Visiting the website of the National Cancer Institute reflects this new standard with the vast majority of clinical trials accepting preoperative chemoradiotherapy before total mesorectal excision as the standard treatment option.

DETERMINATION OF PROGNOSIS

Histopathological Prognostic Markers

The tumor, node, metastases (TNM) classification of solid tumors remains the most powerful indicator of prognosis for colorectal tumors. The extent of the tumor through the bowel wall as well as the infiltration of locoregional lymph nodes is inherently related to patient's survival. At one end, patients presenting with early stage I (T1 and T2 tumors) have an excellent prognosis with surgery alone. In contrast to colon cancer, an intermediate stage II rectal cancer (more advanced T stage:

T3 or T4 without lymph node invasion) is confined with a less good prognosis and requires additional treatment such as radiotherapy with or without chemotherapy. At the other end, the category of stage III tumors (comprising lymph node metastases without distant metastases) bares a high risk of recurrence, but there are subgroups with varying prognosis. Overall survival rates differ between 25% and 55% according to the amount of nodes involved and the depth of invasion into the bowel wall (stages IIIA, IIIB and IIIC) (Greene *et al*., 2004). But even a group of stage III patients with identical TNM displays a wide heterogeneity in risk of recurrence that is still not completely understood.

Furthermore, the number of nodes resected and examined determines prognosis with a minimum of 12 nodes being generally accepted as surgical standard. This observation stresses the need for optimal surgery. It is now recognized that patients with rectal cancer should be referred to specialized multidisciplinary high volume centers. In the past, the local recurrence rate has been linked to the amount of patients operated in one center and by one surgeon per year. High volume centers and very experienced surgeons yield very low local failure rates with improved survival rates (Kockerling *et al*., 1998).

In addition to the T- and N- stages the tumor involvement of the so-called circumferential resection margin (CRM) has been recognized as an important risk factor both for local and distant relapse. CRM is the radial margin that the surgeon includes in the sharp dissection of the mesorectum. A critical distance between the tumor and the CRM of 1 mm appears to be essential for optimal local control and furthermore, a positive CRM (distance ≤1 mm) is associated with an increased incidence of distant metastases (Baik *et al*., 2007). A positive CRM is partly related to insufficient surgery, but may also reflect an advanced stage disease. Importantly, neither preoperative nor additional postoperative radiotherapy does compensate for this R1-resection situation that leaves microscopic tumor foci behind in the pelvis (Baik *et al*., 2007).

Molecular markers that can be determined with immunohistochemical or molecular methods on the paraffin-embedded tissue or on frozen material provide further tools to more precisely determine the individual's risk for relapse. Microsatellite instability (MSI) and mutations in mismatch-repair genes are well-known prognostic markers that confer the patient with a better prognosis. They can readily be determined by immunohistochemistry on paraffin embedded tissue (e.g., the MLH1-, MSH2-proteins) and occur in ~ 15% of sporadic cancers. However, it remains controversial whether MSI-high tumors are associated with a better response to 5-fluorouracil based chemotherapy in colon cancer.

The accuracy of the classical pathological TNM (pTNM) stage relies on the analysis of the surgical specimen. In the era of preoperative treatment modalities, the value of the clinical TNM (cTNM) staging is less clear and the accuracy depends on the clinical staging procedure applied. Even less evident is the role of the ypTNM stage after preoperative treatment and in how far it is consistent with the prognosis that was established by the pTNM classification in nonpretreated patients. The German CAO/ARO/AIO-94 trial indicates that the TNM stage retains its prognostic value even after preoperative treatment.

A retrospective analysis demonstrated that histopathological tumor regression grade according to the Mandard score (4: complete regression, 0: no fibrosis, all viable tumor) as well as the pN-stage were the most important independent prognostic markers for disease-free survival (Rodel *et al*., 2005). A Polish trial that compared preoperative short-term radiotherapy versus preoperative long-term chemoradiotherapy also demonstrated the ypN-stage being the single most important predictor for therapy resistant disease and worse outcome (Bujko *et al*., 2004). The ongoing prospective randomized trials in the field will have to validate how far the ypTNM stage predicts patients' outcome and, e.g., which of these patients need further adjuvant chemotherapy to reduce the risk of relapse.

Pathological Complete Remission

During the last decade, new potent antitumor drugs have increased the rate of pathological complete remissions (pCR) from 2% for 5-fluorouracil alone up to 30% with irinotecan or oxaliplatin based regimens (Aschele *et al*., 2005; Sauer *et al*., 2004). However, the predictive significance of pCR remains unclear, even though many investigators postulate that pCR is a predictive marker of improved patients' outcome similar to what is observed in breast cancer. Unfortunately, most of these reports are retrospective analyses from single center experiences (Stipa *et al*., 2006), and there are currently no prospective data available to confirm this hypothesis. Furthermore, a recent survey of phase II and III trials did not show that pCR reliably predicts late outcome (Glynne-Jones *et al*., 2006). Another single institution report of > 130 patients confirmed that more potent chemoradiation regimens are associated with increased tumor response, however, in the multivariate analysis only pretreatment, clinical T stage and not tumor response were predictors for disease-free survival and overall survival (Pucciarelli *et al*., 2004).

A large monocenter experience reported encouraging results of patients in *clinical* complete remission who did not undergo surgery compared to a group of patients with incomplete clinical remission after preoperative chemoradiotherapy using 5-fluorouracil: 5-year overall survival was 100% in the 71 patients who were not operated compared to 88% in the 22 patients who had a pCR despite the estimation of a clinical incomplete remission (Habr-Gama *et al*., 2004). However, this study harbors several weaknesses: the clinical staging procedure did not correspond to what is considered standard today, no MRI was performed and EUS only in selected cases. Computed tomography (CT) scan only is insufficient to accurately determine clinical tumor stages, particularly after preoperative chemoradiotherapy. Furthermore, distant recurrences occurred in both groups, indicating that local control is not the main issue in this patient population. The authors state that two patients even died from distant metastases in the nonoperative group, but overall survival is reported to be 100%, which is obviously misleading.

In conclusion, the potential of omitting surgery in selected patients who might be cured with modern chemoradiotherapy techniques associated to biological agents (e.g., EGFR- or VEGF-inhibitors) is certainly appealing. In the future, prospective trials including modern imaging techniques such as magnetic resonance

imaging and positron emission tomography (PET)/CT will have to be conducted in order to investigate this nonoperative approach. Avoiding surgery in a subgroup of patients would have a direct impact on quality of life because patients can be spared potential mutilating colostomy procedures and postoperative complications. Nevertheless, the gold standard of tumor staging actually remains the TNM system with the caveat of preoperatively treated patients. However, the standard staging procedure, as determined with the c-, p-, or ypTNM-system still comprises a very heterogeneous cohort of patients. Therefore, new surrogate markers are urgently needed to better discriminate different risk groups in order to offer stage-tailored therapies and to avoid over-treatment.

CLINICAL STAGING

Since the introduction of preoperative multimodal treatment for several gastrointestinal malignancies (e.g., esophagus, rectum), the accurate assessment of disease stage at diagnosis, and thus the patient's prognosis became more difficult. In rectal cancer, only few prospective studies have consequently analyzed the role of the different work-up methods that are now available. The recommendations of preoperative, clinical staging are based on the following data available in the literature.

The aim of preoperative staging is to identify early stage disease (T1 and T2 stages, N0), because these patients only require surgery without additional treatment and thus need to be preserved from over-treatment. In addition to the digital rectal examination, endoscopic ultrasound (EUS) has become part of the standard workup procedure for the diagnosis of rectal cancer because it is one of the most accurate techniques to predict the infiltration into the bowel wall. It allows for initial tumor staging (T-stage) with high accuracy ranging from 70% to 97% especially for early stage cancer (T1-, T2-, non bulky T3-stages) (Beets-Tan *et al*., 2001). Nevertheless, the technique has some limitations; in the case of a stenosing tumor, the probe cannot be placed correctly and furthermore, in rare cases, the examination might be very painful to the patient and thus cannot be performed.

Magnetic resonance imaging is also frequently used in order to detect tumor extending into the surrounding perirectal fat (T3) or adjacent organs (T4) as well as locoregional lymphatic involvement (N+). Additionally, bulky T3 stages can be better visualized with MRI than with EUS and be distinguished from T4 stages. Magnetic resonance imaging is considered a more sensitive technique than CT scan in the delineation of the mesorectal fascia and its potential tumor infiltration (Branagan *et al*., 2004). The involvement of the CRM can be predicted by MRI with an accuracy of > 95%. However, the accuracy of detecting metastatic pelvic lymph nodes remains poor with all three imaging modalities having a sensitivity of 30–60%.

The introduction of modern functional imaging techniques such PET may provide a better tool to identify malignant nodal involvement. The technique that mostly uses ^{18}F-labelled fluoro-2-deoxy-D-glucose (FDG) allows the visualization and quantification of radiolabeled glucose metabolism, and is currently investigated within prospective clinical trials. Furthermore, PET/CT helps at defining target volumes within the planning

of conformal radiotherapy (Patel *et al*., 2007). An additional advantage might be the tumor response prediction to preoperative therapy as it is already established in other gastrointestinal tumors. Applications of various imaging modalities to cancer diagnosis and assessment of cancer therapies are extensively discussed in the recent Handbooks (Hayat, 2007).

Recommendations for Clinical Work-Up

All patients should undergo physical examination and digital rectal examination in order to determine if the tumor is located near the anal verge and whether it is mobile or fixed (potential T4 stage). *CT scan* of the thorax and abdomen indicates the presence of distant metastases. *MRI* of the pelvis should be performed to delineate the CRM and determine the N status. Transrectal EUS is recommended to distinguish between T2 or T3 (or eventual T1 stage) and gives additional information on potential nodal involvement. PET-CT might be offered to patients within investigational studies.

NEW DRUGS TO CONTROL DISTANT METASTASES

The major accomplishment of the last 20 years was to reduce local relapse rate from 40% to 5%, a direct consequence of improved surgical techniques and preoperative therapies. Indeed, the downsizing of the tumor and the sterilization of perirectal tumor deposits can be achieved by preoperative treatments together with TME, reflected by low local recurrence rates. Nevertheless, mortality in this patient population remains too high: 30–40% of patients will ultimatively succumb to their disease. The occurrence of hematogenous, visceral metastases is responsible for this poor overall outcome in an initially potentially curable disease. All larger trials have consistently reported an incidence of distant metastases over 35% and this has not declined despite the improvements in local control (Sauer *et al*., 2004).

5-Fluorouracil and Leucovorin

One of the main reasons for the lack of consistent survival impact of preoperative chemoradiotherapy certainly is the type of chemotherapy used in the landmark trials, as discussed before (Table 24.1). In the past, 5-fluorouracil was the mainstay of chemotherapy because it has been the only available drug with a modest, but significant antitumor activity in colorectal cancer. It had become a cornerstone in the treatment of colorectal cancer since it demonstrated a significantly improved survival when given as adjuvant chemotherapy in resected (stage III) patients as well as in metastatic disease (Poon *et al*., 1991). In the adjuvant setting, the Mayo-clinic regimen of bolus 5-fluorouracil combined with low dose leucovorin (folinic acid, LV) during 6 months or 5-fluorouracil combined with levamisole during 12 months equally prolonged survival by 10% from 66% to 76% in stage III patients (NSABP-C03 trial and IT 0089 trial) (Haller *et al*., 2005; Wolmark *et al*., 1993). Interestingly, increasing the dose of LV did not improve overall outcome. This has been demonstrated in a large randomized trial of > 4,900 patients comparing LV 25 mg/m^2 and LV 175 mg/m^2 associated with bolus 5-fluorouracil with

identical 3-year survival rates of 70% and 71% (Quasar, 2000).

However, in the metastatic setting, overall response rate with the monthly 5-fluorouracil bolus regimen (425 mg/m^2/day × 5) is low, but nevertheless, it allowed for a significant improvement in median survival to > 12 months when associated with low dose leucovorin compared to 5-fluorouracil alone (median survival 7–8 months) (Poon *et al*., 1991). Ever since, LV became an integral part of the 5-fluorouracil based chemotherapy regimens. The protracted i.v., administration of 5-fluorouracil during 24 or 48 h allowed for an enhanced activity of the drug with an improved median progression-free survival from 5 to 7 months (Kohne *et al*., 1998). In all subsequent 5-fluorouracil based trials the protracted infusion schedule combined with leucovorin has been established as the standard way to administer 5-fluorouracil.

Irinotecan

Irinotecan, a camptothecin derivative, was the first agent to be approved by the Food and Drug Administration (FDA) for the concomitant first-line use with 5-fluorouracil/LV for metastatic colorectal cancer in the year 2000. The initial clinical development demonstrated an impressive activity of single agent CPT-11 in 5-fluorouracil resistant disease leading to an approval of the single agent. In a randomized phase III trial the 1-year survival was increased to 36% for patients who were given second-line CPT-11 compared to 14% in the group of best supportive care (p = 0.01) (Cunningham *et al*., 1998). Furthermore, the combination of CPT-11 with 5-fluorouracil as first-line treatment of metastatic colorectal cancer showed a significant survival advantage over 5-fluorouracil alone and the median survival was prolonged from 14 to 17 months (p = 0.031) (Douillard *et al*., 2000). This is also reflected by a significantly higher ORR of 35% in the irinotecan group in comparison to only 22% in the protracted infusion 5-fluorouracil/LV-alone group (p < 0.005).

The camptothecins belong to the class of topoisomerase-I inhibitors that induce enzyme-mediated DNA damage, ultimatively leading to cell death. There is solid preclinical and clinical evidence that they display potent radiosensitization properties. The improved antitumor activity of CPT-11 compared to single agent 5-fluorouracil in metastatic colorectal cancer as well as its radiosensitizing properties made it an attractive agent for a combined chemo/radiotherapy approach in locally advanced rectal cancer despite an overlapping gastrointestinal toxicity. The main ≥grade 3 side effect of CPT-11 is diarrhea in 25% of the patients and, less frequently neutropenia in ~ 10% (Cunningham *et al*., 1998). The latter particularly occurs in patients with a specific polymorphism of the gene encoding the hepatic enzyme uridine-diphosphate-glucuronosyltransferase 1A1 (UGT1A1), which is involved in the metabolism of CPT-11. Patients presenting the genotype **28/*28* (7/7), accounting for ~ 10% of Caucasians, are at higher risk of neutropenia and some oncologists suggest that they should be treated with a lower dose of CPT-11 (Innocenti and Ratain, 2006). But, there is no consensus yet whether all patients should undergo *UGT1A1* genotyping or whether baseline screening of bilirubin levels is sufficient. The **28/*28* genotype has been associated with the benign hyperbilirubinemia *Gilbert* syndrome. The concurrent supportive treatment with loperamide and

eventual hydration as well as a close monitoring of the hematotoxicity enabled a safe administration of CPT-11 together with radiotherapy.

Few trials investigated the addition of CPT-11 to preoperative standard radiotherapy in rectal cancer (45 Gy with 1.8 Gy/fraction over 5 weeks), with or without 5-fluorouracil or capecitabine in the last 2–5 years (Hofheinz *et al*., 2005; Klautke *et al*., 2005). All demonstrated good feasibility with an acceptable toxicity profile and promising activity. We have demonstrated that CPT-11 at 90 mg/m^2/week × 3 given together with hyperfractionated accelerated radiotherapy was well tolerated in our recently published trial (Voelter *et al*., 2006). The incidence of severe diarrhea was 24%, comparable to standard 5-fluorouracil based chemoradiotherapy: only recently the RTOG 0012 phase II trial that randomized chemoradiotherapy with 5-fluorouracil and CPT-11 reported an incidence of diarrhea of 28% in patients who were given 5-fluorouracil compared to 37% treated with CPT-11 (Mohiuddin *et al*., 2006).

The introduction of potent systemic agents early in the treatment course is based on the rationale of enhancing radiosensitivity and eliminating eventual micrometastatic disease. However, while local control in rectal cancer patients has been substantially improved during the last decade, patients still die because of distant metastases. Actual trials in the field of rectal cancer need to aim at diminishing the incidence of distant metastases in order to improve patients' outcome. The cumulative dose of CPT-11 of 270 mg/m^2 in our trial accounted for 80% of the recommended dose per cycle in the single agent setting without radiotherapy. Nevertheless, this dose failed to prevent the development of distant metastases in 34% of patients despite the postoperative, adjuvant administration of CPT-11 based chemotherapy (Voelter *et al*., 2006). The radiotherapy regimen has now been adapted to a prolonged schedule in the subsequent study (1.8 Gy per fraction per day for a total of 45 Gy), allowing for an increase of preoperative chemotherapy exposure, since the dose of CPT-11 cannot be further increased in combination with hyperfractionated accelerated radiotherapy. The feasibility of the combination CPT-11 and radiotherapy being established, future trials aim at adding novel targeted compounds into preoperative chemoradiotherapy regimens.

Cetuximab

Cetuximab is a targeted therapeutic agent, a chimeric IgG1 monoclonal antibody that specifically binds to the EGFR with high affinity. The EGFR is a transmembrane glycoprotein, which is commonly expressed in many normal human tissues (e.g., the skin). It was one of several growth factors and their receptors, which were found to be encoded by proto-oncogenes. It is a member of the tyrosine kinase family of growth factor receptors, and is overexpressed in many human tumor types, e.g., in > 50% of rectal carcinomas. Preclinical evidence suggests that tumor cells with a high degree of EGFR expression proliferate more, probably due to activation via an autocrine pathway. Furthermore, EGFR overexpression confines the tumor cells with a survival advantage through mechanisms related to enhanced cell growth and division.

EGFR antagonists have been developed in order to inhibit proliferation of EGFR-expressing cells. They block the ligand-

binding site and lead to internalization of the receptor and prevent the physiological ligands EGF and TGF-α from interacting with the receptors, and thus effectively block ligand-induced EGFR phosphorylation and downstream signaling (Huang *et al*., 1999). The rationale of integrating EGFR antagonists in the treatment of rectal cancer is two-fold: First, ionizing radiation has been shown to activate the EGFR signaling cascade via direct induction of receptor dimerization. Furthermore, EGFR overexpression has been associated with radioresistance in preclinical *in vivo* and clinical studies (Baumann and Krause, 2004). In a retrospective study of 76 patients treated with preoperative chemo/radiotherapy the overall pathological response rate was almost double in EGFR-negative tumors (62%) compared to EGFR-positive tumors (34%) (Giralt *et al*., 2005), and the observation was confirmed in other trials. It has been postulated that EGFR positive clonogenic tumor cells are confined with inherent cellular radioresistance as well as a potentially lower oxygenation status and additionally these cells might provide a more favorable protective tumor microenvironment (Baumann and Krause, 2004).

Secondly, EGFR inhibitors such as cetuximab act as a radiosensitizer in preclinical models. In a model of human tumor xenografts in mice the injection of cetuximab led to significantly reduced tumor growth when combined with irradiation. This was recently confirmed within clinical trials: cetuximab increases the efficacy of radiotherapy in head and neck cancer patients with a significantly prolonged survival (Bonner *et al*., 2006). Based on this radiobiological rationale together with the knowledge of a synergistic activity of cetuximab and CPT-11 shown in colon cancer the triple preoperative association of radiotherapy, chemotherapy with CPT-11 and the biological agent cetuximab has entered clinical trials in rectal cancer.

Furthermore, the integration of novel therapies such as cetuximab early in the treatment course has the potential to eradicate micrometastatic disease. Several actual clinical trials investigate the association of cetuximab, CPT-11 with or without a fluoropyrimidine in association with preoperative radiotherapy in rectal cancer. Recently, the results of a pivotal phase I study have been become available investigating the triple association capecitabine (an oral fluoropyrimidine), CPT-11 and cetuximab together with radiotherapy (Hofheinz *et al*., 2006). The combination displayed promising activity with a pathological complete remission rate of 25%.

Oxaliplatin and Bevacizumab

Oxaliplatin is a platinum-derivative that has a different toxicity profile than cisplatinum: neither nephrotoxicity nor ototoxicity, but more frequent neurotoxicity. During the clinical phase I and II studies it has been found to be effective in several gastrointestinal cancer types. Its particular promising activity in metastatic colorectal cancer (de Gramont *et al*., 2000) led to the approval by the FDA in 2002 for the use in combination with infusional 5-fluorouracil. Like all platinum-derivatives, oxaliplatin displays radiosensitizing properties and has been investigated in preoperative chemoradiotherapy trials together with 5-fluorouracil. Preliminary results from phase I and II trials indicate that the combination yields

promising antitumor activity with pathological complete remission rates between 14% and 28% (Aschele *et al*., 2005). The incidence of treatment induced severe diarrhea as well as the rate of postoperative complications is comparable to 5-fluorouracil and CPT-11 based regimens; however, neurotoxicity is an inherent side effect of oxaliplatin and a grade 2 occurs in over half of the patients treated.

Bevacizumab is a humanized monoclonal antibody that targets the soluble vascular endothelial growth factor VEGF. Angiogenesis is a critical phenomenon during tumor growth and VEGF is the main transmitter of proangiogenic effects by increasing vascular permeability, endothelial cell activation, tumor cell migration, invasion and proliferation. Indeed, VEGF overexpression has been associated with inverse prognosis in colorectal cancer patients (Altomare *et al*., 2007). Preoperative chemoradiotherapy seems to induce increased VEGF levels and therefore, VEGF inhibitors are ideal drugs to be integrated within combined preoperative treatment regimens.

Preclinical models have shown that bevacizumab acts as a radiosensitizer; however, the exact mechanism of action is not completely understood. Several hypotheses claim that the antitumor effect is mediated through radiosensitization of tumor associated endothelial cells and thereby preventing neovascularization. Furthermore, bevacizumab induces a normalization of the vasculature and the microenvironment, thus confining the tumor with better oxygenation. Lastly, bevacizumab is supposed to directly target circulating endothelial cells (Willett *et al*., 2006).

Indeed, the first clinical study in rectal cancer that associated bevacizumab with continuous 5-fluorouracil and preoperative radiotherapy in a phase I trial revealed evidence for tumor vasculature normalization *in vivo* and a high rate of pCR (Willett *et al*., 2006). However, perioperative toxicity with an increased incidence of bleeding and thrombovascular events is a major concern and has to be closely monitored. Due to the long half-life of the antibody a minimum of a 5 weeks-gap without treatment has to be assured before surgery. The best way to integrate this potent antiangiogenic agent within current chemoradiotherapy regimens has yet to be established.

NEW PREDICTIVE MARKERS

The heterogeneity within a given histopathological prognostic group of patients remains a matter of debate. Additional biomarkers that are able to readily predict response to chemoradiotherapy are needed to better discriminate patients at high risk of relapse who would benefit from additional (preoperative and/or postoperative) treatment. Presently, no predictive tissue or soluble biomarkers have been established and validated neither in colon nor in rectal cancer.

Recently, the European trial on adjuvant CPT-11 and 5-fluorouracil in colon cancer accomplished accrual of 3,005 patients and tumor tissue was collected in > 1,500 patients. This collection enables for the first time a large-scale prospective analysis of biomarkers in colon cancer, and preliminary results were reported at the ASCO meeting 2007 (Roth *et al*., 2007). Overexpression of the p53 protein as well as thymidylate synthetase (TS), MSI-high tumors and *Kras* mutation were observed

in 37%, 48%, 15% and 37% respectively. However, correlation with disease-free survival is only available for MSI and SMAD4 expression at time of elaboration of the present manuscript, confirming an improved prognosis for MSI-high tumors and an impaired prognosis for tumors lacking SMAD4 expression, a marker associated with 18q deletion.

With regard to rectal cancer, the data in the literature are less robust with mainly small and retrospective series reported. However, several markers of interest have evolved from these studies that might be suitable for prospective analysis, e.g., thymidilate synthase (TS) that is a protein implicated in the metabolism of 5-fluorouracil. In the past, it has been suggested that TS expression is a predictive marker for survival in patients treated with 5-fluorouracil based chemoradiation with high levels of TS expression being associated with worse survival. However, TS overexpression in the tumor seems to be correlated with better response to adjuvant 5-fluorouracil in terms of prolonged disease-free survival and overall survival (Johnston *et al*., 1994).

EGFR Signaling Pathway

The most recent acknowledged protein is at the same time one of the best studied molecules and it belongs to the family of receptor tyrosine kinases: the receptor of the EGF mediated cell signaling pathway, EGFR. The absence of EGFR expression has been described as a positive prognostic factor in rectal cancer, particularly when EGFR staining was negative after preoperative chemoradiation (Giralt *et al*., 2005). However, more than half of the patients will have EGFR-positive rectal cancer at diagnosis and thus are at high risk of relapse. The determination of EGFR status has the potential for distinguishing patients at high risk of relapse on the one hand, and on the other hand, these patients might benefit from EGFR-targeted therapies, such as anti-EGFR monoclonal antibodies (e.g., cetuximab, panitumumab).

Mutations of *Kras*, an oncogene that is involved in the downstream signaling of the receptor tyrosine kinase family such as EGFR, have been shown to correlate with the probability of tumor response to EGFR inhibitors. In a series of 59 colorectal cancer patients treated with chemotherapy and cetuximab none of the tumors of responding patients harbored a *Kras* mutation (Di Fiore *et al*., 2007). The mutated *Kras*, which occurs in ~ 37% of colorectal tumors, therefore, seems to confer the tumor cells with resistance to EGFR-inhibitor based treatments. Nevertheless, the relationship between EGFR overexpression and *Kras* mutation has not yet been established.

Cell Cycle Associated Proteins and Circulating Tumor Cells

Cell cycle associated and DNA repair genes (encoding for mismatch repair enzymes MMR) are inherently connected to chemotherapy and radiation induced cell damage, and represent potential biomarkers to be investigated within prospective clinical trials. Cell cycle associated molecules such as p21 and p53 have been proposed as independent prognostic factors for survival in rectal cancer patients. The cyclin-dependent inhibitor p21 delays the progression from G1 to S phase, and thereby prevents the replication of damaged DNA. Overexpression of p21 in rectal cancer has been described to enhance radiosensitivity. In contrast, mutations of

p53, a tumor suppressor gene mediating cell cycle arrest and apoptosis, is recognized to be an important element for radioresponse and has been linked to impaired survival (Zlobec *et al*., 2005). Recently, a well-conducted prospective monocenter study demonstrated that a combination of a standard histopathological marker (pN stage) and the determination of Cyclin D1 gene polymorphism accurately distinguished subgroups of patients at higher risk of disease recurrence (Ho-Pun-Cheung *et al*., 2007).

In another trial of 67 rectal cancer patients treated with preoperative CPT-11 and 5-fluorouracil combined with radiation, p21 was expressed in all tumors with a pathological complete remission (pCR) at the time of surgery (Mitchell *et al*., 2003). In the same study, MSI was a predictor for tumor regression with all MSI-high tumors being in pCR.

The detection of circulating tumor cells using molecular techniques such as polymerase chain reaction (PCR) for cell surface epitopes might be an elegant method in the future to determine patients at high risk of relapse after preoperative treatment (Kienle *et al*., 2003). In a series of > 120 patients with locally advanced rectal cancer, circulating tumor cells were determined with cytokeratin 20-reverse transcription PCR in blood and bone marrow samples. The detection rate was lower in patients who had undergone preoperative chemoradiotherapy, and patients with persistent circulating tumor cells had worse disease-free and overall survival. However, this study included 25 patients in the preoperative treatment arm versus 117 patients who were operated only. Certainly, the hypothesis of reducing circulating tumor cells by means of preoperative chemoradiotherapy is very elusive; however, larger well-powered prospective trials have to be conducted to confirm this observation.

Additionally, genomic profiling of multiple genes may allow for a more precise individual risk assessment. The first reports indicate that, e.g., gene polymorphisms of interleukin-8 are individually associated with risk of recurrence (Gordon *et al*., 2006). Based on these examples of mainly small retrospective analyses future prospective trials might integrate e.g., genomic profiling and determination of circulating tumor cells in the risk stratification of patients. In summary, translational projects must be an integral part of every clinical therapeutic trial in rectal cancer in the future with the prospective sampling of biological material, such as tumor and blood in order to establish risk-tailored treatment strategies.

UNSOLVED QUESTIONS IN THE TREATMENT OF RECTAL CANCER

One remaining question involves the optimal time interval between the end of preoperative chemo/radiotherapy and surgery. Many radio/oncologists and surgeons advocate that a longer interval favors the protracted downstaging effect induced by the preoperative treatment. However, there never has been a well-designed randomized trial investigating a comparison between the short interval as studied in the Swedish and Dutch trials (Table 24.1) and a longer interval that is generally applied (e.g., in the German study). Two trials are often cited in this context; however, neither had been designed to solve

this question: The Polish trial compared a radiotherapy alone group followed by a short interval with a chemoradiotherapy long interval group and thus the treatment arms were not directly comparable (Bujko *et al*., 2004). Another French trial concluded that a long interval is superior to a short interval, but once again, the primary endpoint of the trial (increase of sphincter sparing surgery in the long interval group) was not met (Francois *et al*., 1999). The assumption that long interval should be considered as standard was based on the observation that the pathological response rate was increased in the long interval group in this trial despite no effect on local or distant relapse rate.

However, as mentioned in another chapter, there is no evidence from the literature that downstaging of the primary tumor (T-stage) or the rate of pathological complete remissions represent a valid surrogate for disease recurrence (Pucciarelli *et al*., 2004). Additionally, despite the observation that T-downstaging is achieved by preoperative treatment modalities the incidence of positive lymph nodes (pN+, stage III) at time of surgery has remained unchanged between 30–50% (Kapiteijn *et al*., 2001; Sauer *et al*., 2004).

To date, no valid recommendation can be formulated with regard to the interval that has to be applied between the end of radiotherapy and surgery, except in case of short-term radiotherapy according to the Swedish and Dutch trials, where surgery should be performed after 1 week. In current practice, however, an interval of 3–6 weeks is generally adopted after long-term chemo/radiotherapy regimens. Once, the optimal preoperative chemo/radiotherapy regimen is established, a prospective trial has to be conducted to definitely investigate the influence of the timing-issue on patients' outcome.

Similarly to the uncertainty of the prognostic value of ypTNM stage, as discussed in another chapter, the role of adjuvant chemotherapy for patients who already had received preoperative systemic therapy remains a matter of debate. In the future, the results of ongoing trials will shed more light on the role of ypTNM stage and the necessity of further postoperative chemotherapy (eventually with a biological agent) for certain subgroups of patients.

In conclusion, much progress has been made during the last 3 decades in the treatment of locally advanced rectal cancer patients. Local recurrence rates have dropped dramatically from 40% to under 10%. Large training programs have enabled an improvement of surgical techniques and TME has now become the standard operating procedure. Despite TME, preoperative radiotherapy has been proven to further increase local control for rectal cancer. Furthermore, combined chemoradiotherapy administered before surgery has become the standard treatment option for locally advanced tumors worldwide. New antitumor agents have enriched the systemic therapies and these can be readily associated to radiotherapy with the potential to target micrometastatic disease early in the treatment course. Indeed, the fight against disease relapse at distant sites remains the most important and most urgent unsolved problem in the beginning of the 21st century.

Correct staging of the tumor in the preoperative situation remains a challenge and new imaging techniques might improve on the accuracy of clinical staging. With the amelioration of up-front chemoradiotherapy

with potent systemic agents such as the cytostatics CPT-11 and oxaliplatin and the biological agents, e.g., cetuximab and bevacizumab a new debate on the possibility of a nonoperative approach might evolve in future decades.

Meanwhile, efforts have to be undertaken to improve on clinical research including translational research. The aim is to determine new surrogate biomarkers that may help to distinguish different groups of tumors and patients at higher risk of relapse and thus to offer real stage-tailored treatments and to avoid overtreatment.

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Locally Advanced Rectal Cancer: Combined Chemotherapy During Preoperative Radiation Therapy

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INTRODUCTION

Since the early 1990s, a combined modality approach, consisting of surgery, pelvic irradiation, and chemotherapy with 5-fluorouracil (5-FU), has been the recommended management for patients with operable stage II and III, i.e., locally advanced rectal cancer. However, the last 15 years have brought forth a great improvement in the management of locally advanced rectal cancer that has challenged the 1990 consensus statement.

Advances in surgical techniques, in particular the increased accomplishment of total mesorectal excision, have led to a decline of local recurrence rates. Moreover, pelvic radiation therapy, even in the setting of an optimal surgery, has been demonstrated to further reduce the risk of local recurrence. However, adjuvant radiation therapy has shown no benefit on overall survival.

On the other hand, 5-FU-based chemotherapy has admittedly led to a significant improvement of 5-year overall survival. Most recently, prospective data have suggested that a preoperative combined modality therapy may increase the pathologic downstaging (increasing the opportunity of performing sphincter-preserving surgery), and is also associated with a lower incidence of acute toxicity compared to the postoperative combined approach. Furthermore, improvements in imaging methods, such as high resolution magnetic resonance imaging, may provide more accurate staging information of paramount significance in selecting patients for neoadjuvant therapy, identifying patients in whom more aggressive treatment might further increase the cure rate.

Despite the management of locally advanced rectal cancer with combined modality therapy that seems to have remarkably improved local recurrence, the risk of distant metastases remains a significant problem. Therefore, research interest has now switched to determine the optimal combination of cytotoxic agents, delivery in conjunction with pelvic radiation therapy and surgical resection with total mesorectal excision, to patients with locally advanced rectal cancer at high risk of (distant) recurrence. In this chapter, the use of chemotherapy as part of a combined modality approach for locally advanced rectal cancer will be examined.

CHEMOTHERAPY OF LOCALLY ADVANCED RECTAL CANCER

Given the overall survival results reported by two prospective randomized North American trials (the Gastrointestinal Tumor Study Group [GITSG]-7175, and the North Central Cancer Treatment Group [NCCTG]-794751), the National Institutes of Health Consensus Conference (1990) concluded that chemo-radiotherapy should be the standard postoperative adjuvant treatment for patients with T3-T4 and/or N1-N2 rectal cancer.

The four-arm GTSG-7175 study randomly assigned 227 patients with rectal cancer, after curative surgical resection, either to observation, to pelvic radiation therapy, to chemotherapy (5-FU plus semustine), or to a combination of pelvic radiation therapy and chemotherapy. After a median follow-up of 80 months, a pairwise comparison demonstrated a significant reduction of recurrence rate (33% *vs.* 55%), and a statistically significant advantage for disease-free survival, and overall survival only for patients receiving combined radiation therapy and chemotherapy compared with the untreated control ($P < .04$ and $P < .009$, respectively).

In the two-arm NCCTG-794751 study, 204 patients with operable rectal cancer were randomly assigned to receive postoperatively either pelvic radiation therapy alone or radiation therapy combined with chemotherapy (5-FU and semustine). After a median follow-up of more than 7 years, there was a 34% reduction in tumor relapse ($P = .002$), and a statistically significant increase of disease free survival ($P = .0016$) and overall survival ($P = .025$) in favor of the combined modality treatment. However, considering that the GTSG-7175 study was statistically underpowered, and that in the NCCTG-794751 study both groups received pelvic radiation therapy, neither of these two trials was conclusive on whether radiation therapy and chemotherapy, when used in combination, had an additive effect on overall survival.

This question was dealt with by the National Surgical Adjuvant Breast and Bowel Project (NSABP) trials, demonstrating that 5-FU-based chemotherapy was the key component in improving the overall survival. In the NSABP R01 trial, reported by Fisher *et al.* (1998), 555 patients with locally advanced rectal cancer treated by curative resection were randomly assigned to receive no further treatment, postoperative pelvic radiation therapy, or postoperative chemotherapy with 5-FU, semustine and vincristine (MOF regimen). After 5 years of follow-up, there was a statistically significant advantage in disease free survival (42% *vs.* 30%, P = .006) and overall survival (53% *vs.* 43%, $P = .05$) in favor of the group of patients that received postoperative adjuvant chemotherapy. These findings were supported by the following NSABP R02 trial, reported by Wolmark *et al.* (2000), that randomized 694 locally advanced rectal cancer patients, addressing whether the combination of radiation therapy and chemotherapy (either 5-FU plus leucovorin or MOF) would enhance the survival advantage observed in the trial NSABP R01. Although patients assigned to receive radiation therapy and chemotherapy had a significantly reduced local recurrence compared with patients receiving chemotherapy alone (8% *vs.* 13%, $P = .02$), no benefit in disease free survival ($P = .90$) nor in overall survival ($P = .89$) was documented by the combined treatment. Moreover, the results of the NSABP trial

R02 underlined the efficacy of 5-FU plus leucovorin, but confirmed an increase of acute toxicity, as reported in previous trials: in the postoperative combined approach, nearly 40% of patients experienced severe toxicity (mainly diarrhea), and about 30% of patients did not complete the planned chemotherapy with 5-FU plus leucovorin.

Therefore, a preoperative approach has been pursued to reduce the acute and late toxicity based on the following rationale: with primary radiation therapy there is no irradiation of the anastomotic region, much of the irradiated bowel is removed at the time of surgery, and the small bowel is not fixed in the pelvis as a consequence of post-surgical adhesion. Other potential advantages of the preoperative approach include: decreased tumor seeding, increased radio-sensitivity due to more oxygenated cells, enhanced sphincter preservation in low-lying rectal cancer, and the possibility to convert to resectability a tumor not amenable to a curative resection at presentation. Conversely, the primary disadvantage of this strategy is the risk of over-treating patients with early (pathologic T1–2N0) disease.

Furthermore, many European investigators have advocated the preoperative approach based on the results of a Swedish trial, suggesting an unique biologic role of radiation therapy in the preoperative setting. The Swedish Rectal Cancer Trial (1997) randomly assigned 1,100 patients with clinically resectable stage T1–T3 rectal cancer to surgery alone versus hypofractionated (short course) preoperative pelvic radiation therapy (25 Gy in five fractions) followed by surgery. At a 5-year follow-up, the group receiving radiation therapy experienced a lower rate of local recurrence (11% *vs.* 27%, $P < .001$), and a significant improvement of overall survival (58% *vs.* 48%, $P = .004$), which was similar to that reported by the chemotherapy arm of the NSABP R01 trial or in the chemo-radiation therapy arm of the GTSG-7175 and NCCTG-794751 trials. However, it should be noted that the Swedish Rectal Cancer Trial patients with clinical T1-2 disease were also enrolled. Moreover, surgery was not standardized, and patients did not uniformly undergo total mesorectal excision, and there was a selection bias, because a statistically greater proportion of patients with a more favorable Dukes' stage was allocated in the radiation therapy arm.

More recently, Kapiteijn *et al.* (2001) reported the results of the Dutch CKVO 95-04 trial, in which 1,805 patients with clinically resectable T1-3 disease were randomized to surgery alone with total mesorectal excision, or to a short-course preoperative pelvic radiation therapy, followed by 1 week later total mesorectal excision. This study failed to confirm any survival benefit with preoperative radiation therapy. Although radiation therapy significantly decreased the local recurrence (8% *vs.* 2%, $P < .001$), there was no difference in 2-year overall survival (81.8% *vs.* 82%, $P = .84$), likely due to the fact that radiation therapy was unable to reduce the occurrence of distant metastases (16.8 *vs.* 14.8%, $P = .87$).

The results of the Dutch trial, that had for the first time the merit of including an extensive assurance program to assess the quality of surgery and radiation therapy, supported the local recurrence benefit of preoperative radiation therapy, even in the setting of an optimal surgery. However, it should be noted that preoperative short-course radiation therapy was followed immediately by surgery, although considered as biologically equivalent to a long-course radiation

therapy (45–50.4 Gy), has the drawback of not causing tumor shrinkage, and therefore it is unable to allow for sphincter preservation in patient candidates for abdominoperineal resection (APR). In addition, a short-course radiation therapy does not allow for a combination with an effective systemic chemotherapy treatment. For these reasons, the use of chemotherapy, also in the preoperative setting, has been pursued in combination with long-course pelvic radiation therapy.

In the early 1990s, encouraging results with long-course pelvic radiation therapy combined with chemotherapy were reported in small non-randomized studies for locally advanced rectal cancer patients. Minsky *et al*. (1992a) reported a higher resectability (90% *vs.* 64%), a greater pathologic complete response rate (20% *vs.* 0%), and a lower incidence of positive nodes (30% *vs.* 64%) in patients with unresectable rectal cancer who received preoperative radiation therapy and 5-FU/leucovorin compared with patients who received radiation therapy alone. Patients with unresectable disease treated with the combined modality approach had a higher pathologic complete response (20% *vs.* 6%), and a lower occurrence of positive nodes (30% *vs.* 53%), even when compared with patients affected by resectable disease treated with radiation therapy alone. Moreover, Minsky *et al*. (1992b) suggested that patients treated with preoperative chemo-radiation therapy were able to tolerate higher chemotherapy doses, and experienced significantly less acute toxicity, compared with the postoperative combined modality. These initial findings, and the availability of more accurate preoperative staging procedures, led to conduct some randomized phase III trials of preoperative versus postoperative chemo-radiation therapy to assess the potential value of the preoperative approach with regard to sphincter preservation, toxicity, local recurrence, and overall survival.

Three randomized clinical trials directly compared the efficacy of preoperative and postoperative chemo-radiation therapy in patients with resectable locally advanced rectal cancer. All trials used 5-FU-based chemotherapy, and mandated that the type of resection should be declared before the preoperative approach. Unfortunately, a low accrual caused an early closure of two of these trials (Radiation Therapy Oncology Group [RTOG] 94-01/Intergroup [INT] trial 0147, and NSABP trial R03). Therefore, the results of the CAO/ARO/AIO 94 trial, reported by Sauer *et al*. (2004) have assumed a special relevance. This trial randomly assigned 823 patients with resectable stage II and III rectal cancer to the same preoperative or postoperative regimen of chemo-radiation therapy, based on conventional long-course pelvic radiation therapy (50.4 Gy in 28 fractions of 1.8 Gy), and concurrent continuous infusion 5-FU during the first and fifth week of radiation therapy. Surgery was standardized with a total mesorectal excision technique, and was performed 6 weeks after chemo-radiation therapy. In the preoperative arm, four cycles of bolus 5-FU, five times a week every 4 weeks, were administered 1 month after surgery, while these four cycles were administered after chemo-radiation therapy in the postoperative arm. Sauer *et al*. (2004) provided evidence of the superiority of the preoperative over postoperative treatment, showing a statistically significant increase of pathologic complete response (8% *vs.* 0%), and a greater sphincter-preservation surgery in

the subgroup of patients with low-lying tumors requiring an abdomino-perineal resection (39% *vs.* 19%). Furthermore, in the preoperative chemo-radiation therapy group, they reported a better compliance with the planned treatment, with a significantly higher number of patients completing the planned radiation therapy (92% *vs.* 54%) and chemotherapy (89% *vs.* 50%), and a significantly lower occurrence of severe acute (27% *vs.* 40%) and late (14% *vs.* 24%) toxicity. Moreover, with a median follow-up of nearly 4 years, the cumulative 5-year local recurrence was less than half in the preoperative compared with the postoperative group (6% *vs.* 13%, P = .006), although significantly more patients in the preoperative group had a low rectal tumor, which is a poor risk factor for local recurrence. However, the lower local recurrence of the preoperative treatment was not associated with a reduction of distant metastases (36% *vs.* 38%, P = .84), and did not translate into a significant improvement of 5-year overall survival (76% *vs.* 74%, P = .80). Of note, this trial did show the greater risk for the preoperative approach of overtreating an early-stage tumor, despite a baseline assessment with endorectal ultrasonography. Indeed, a pathologic stage I was found in 18% of patients randomly assigned to postoperative chemo-radiation therapy.

Whether the addition of chemotherapy to preoperative long-course pelvic radiation therapy is more effective than preoperative radiation therapy alone was addressed in two randomized European trials: the European Organization for the Research and Treatment of Cancer (EORTC) trial 22921, and the Fédération Francophone de Cancérologie Digestive (FFDC) trial 9203. In contrast with the German trial, in both these studies a staging with EUS was optional, and 5-FU bolus during pelvic radiation therapy (for a total dose of 45 Gy) was used. Moreover, although total mesorectal excision was recommended, no central quality assurance was performed. In the EORTC trial 22921, 1011 patients with T3-4 resectable rectal cancer were randomized in a 2 × 2 factorial design to preoperative radiation therapy, with or without concurrent bolus 5-FU/leucovorin, delivered in two 5-day courses during the first and fifth weeks of radiation therapy, followed by surgery, with or without four cycles of postoperative bolus 5-FU/leucovorin, administered with the same schedule as in the preoperative setting. The final report of this trial by Bosset *et al*. (2006) showed that preoperative chemotherapy did not affect the compliance of preoperative radiation therapy, the adherence to postoperative chemotherapy, the feasibility of surgery, and the rate of postoperative complications. Moreover, they reported a significant enhancement of tumor and nodal downstaging by adding 5-FU/leucovorin to preoperative radiation therapy, with an increased pathologic complete response rate (14% *vs.* 5.3%, P < .0001). However, these effects were not associated with a significant increase of sphincter-preserving surgery. These investigators further showed a significant decrease in 5-year local recurrence in the combined preoperative arm (8.7% *vs.* 17.1%, P < .0016), but the 5-year occurrence of distant metastases, disease free survival and overall survival did not differ significantly. Moreover, there was a markedly reduced adherence to postoperative chemotherapy, with less than 50% of patients receiving 5-FU/leucovorin as planned. Finally, although there was a trend for a higher 5-year disease free

survival (58.2% *vs.* 52.2%) and overall survival (67.2% *vs.* 63.2%), these differences were not statistically significant. Interestingly, postoperative chemotherapy compensated for the lack of preoperative chemotherapy on local recurrence (8.7% for preoperative chemo-radiation therapy with no postoperative chemotherapy *vs.,* 9.6% for preoperative radiation therapy followed by postoperative chemotherapy). On the whole, the 5-year local recurrence was 17.1% for patients who did not receive any chemotherapy, whilst it was about 8% for those who received some chemotherapy at any time.

In the FFCD 9203 study, 762 patients with T3-4 resectable rectal cancer were randomly allocated to either preoperative pelvic radiation therapy alone or preoperative concurrent chemo-radiation therapy. Patients in both arms were scheduled to receive postoperative chemotherapy, and the dosage and schedule of radiation therapy and chemotherapy were the same as in the EORTC 22921 trial. The results described by Gérard *et al*. (2006) were similar to those of the EORTC 22921 trial. The addition of 5-FU/leucovorin to preoperative radiation therapy produced only a moderate increase of grade 3–4 acute toxicity, whereas the adherence to postoperative chemotherapy was poor. A significant increase in pathologic complete response rate (11.7% *vs.* 3.7%, $P < .0001$), and a significant improvement in local recurrence (8% *vs.* 16.5%, $P = .004$), which did not modify sphincter preservation at surgery, were observed in the preoperative chemo-radiation therapy arm. Once again, there was no difference in 5-year disease free survival and overall survival between the two arms.

A third randomized Polish trial has evaluated whether preoperative conventionally fractionated pelvic radiation therapy combined with chemotherapy could offer an advantage in sphincter preservation in comparison with preoperative short-term radiation therapy. In this trial, 312 patients with resectable T3-4 rectal cancer were randomized to receive either short-course pelvic radiation therapy alone, or long-course pelvic radiation therapy with bolus 5-FU/leucovorin, followed by surgery with total mesorectal excision. However, there was no central quality assurance for total mesorectal excision, and postoperative chemotherapy was optional. Bujko *et al*. (2006) reported that, despite a significant improvement in pathologic complete response rate (16% *vs.* 1%), the addition of chemotherapy did not increase the rate of sphincter preservation (58% *vs.* 61%, $P = .57$), suggesting that sphincter-saving surgery is mainly related to the surgeon's technical ability. Moreover, similarly to the EORTC 22921 and FFCD 9203 trials, they observed that the downstaging effect of chemo-radiation therapy did not translate in a significant survival benefit, but, unlike these trials, it also did not significantly improve the local recurrence. In this respect, it should be noted that postoperative adjuvant chemotherapy was more frequently used in the short-course radiation therapy arm (47% *vs.* 31%, $P = .006$), as a consequence of the downstaging effect of preoperative chemo-radiation therapy. This difference represented a confounding factor, in consideration of the capability of postoperative chemotherapy to compensate for the lack of preoperative chemotherapy on local failure, as evidenced by the EORTC 22921 trial. Moreover, the short-course radiation therapy arm included

TABLE 25.1. Randomized trials of preoperative and/or postoperative chemoradiotherapy for rectal cancer.

Trials (no. patients)	Arms	Local failure rate (%)	Distant metastasis (%)	DFS (%)	OS (%)
GITSG 7175 (227)	Surgery	24	34	46	45
	Surgery → RT	20	30	52	52
	Surgery → 5FU/MeCCNU	27	27	54	56
	Surgery → RT + 5FU/MeCCNU	11*	26	70*	59
NCCTG/Mayo 794751 (204)	Surgery → RT	25	46	38	48
	Surgery → RT + 5FU/MeCCNU	13*	29*	58*	58*
NSABP R-01 (555)	Surgery	25	26	30	43
	Surgery → RT	16*	31	35	41
	Surgery → RT + MOF	21	24	41*	53*
NSABP R-02 (694)	Surgery → CH	13	29	50	64
	Surgery → RT + CH	8*	31	50	64
CAO/ARO/AIO-94 (823)	Surgery → RT/5FU + FU bolus four cycles	13	36	68	76
	RT/5FU → Surgery → FU bolus four cycles	6*	38	65	74
EORTC 22921 (1,011)	RT → Surgery	17	38		
	RT → Surgery → 5FU/LV four cycles	10*	34	54	65
	RT/5FU → Surgery	9*	35	56	66
	RT/5FU → Surgery → 5FU/LV four cycles	8*	31		
FFCD 9203 (762)	RT → Surgery → 5FU/LV four cycles	16	NA	56	68
	RT/5FU → Surgery → 5FU/LV four cycles	8*		59	67
Polish (312)	RT (5 × 5) → Surgery → 5FU/LV (optional)	11	31	59	67
	RT/5FU → Surgery → 5FU/LV (optional)	16	35	56	66

All reported outcomes but Polish trial (4 year) are at 5 year.

GTSG, Gastrointestinal Tumor Study Group; NCCT, North Central Cancer Treatment Group; NSABP, National Surgical Adjuvant Breast and Bowel Project; CAO/ARO/AIO, German Rectal Cancer Group; EORTC, European Organization for the Research and Treatment of Cancer; FFCD, Fédération Francophone de Cancérologie Digestive; CH, chemotherapy; RT, radiotherapy; MeCCNU, semustine; 5FU, 5fluorouracil; MOF, semustine, vincristine, and 5fluouracile; LV, leucovorin; DFS, disease-free survival; OS, overall survival; NA, not available.

*A significant p value of 0.05 or less in favour of that arm.

a higher rate of pT1/T2 tumors, and the trial's accrual was underpowered to detect small differences between the two arms.

Recently, investigators at the Royal Marsden Hospital have advocated the addition of neoadjuvant chemotherapy before

preoperative chemo-radiation therapy, with the aim of preventing early dissemination of micrometastases, and reducing the radiation field by a chemotherapy-induced tumor shrinkage. Chau *et al*. (2006) reported the results of a phase II study, in which 77 poor-risk rectal cancer patients, defined on the basis of high resolution MRI, were treated with four cycles of capecitabine plus oxaliplatin before preoperative chemo-radiation therapy, including capecitabine and high dose (54 Gy) pelvic radiation therapy followed by surgery, and 12 further weeks of adjuvant capecitabine. After the primary combination chemotherapy, a rapid symptomatic improvement, and a clinical response, assessed by magnetic resonance imaging in 88% of patients was observed. Moreover, a pathologic complete response rate in 21% and only microscopic tumor foci in an additional 48% of patients was found after surgery. However, this approach determined an unpredictable rate of toxic deaths (5%). Therefore, primary chemotherapy before chemo-radiation therapy should be used with caution and restricted to clinical trials.

In summary, the findings of the last trials support the relevant role of chemotherapy in the management of locally advanced rectal cancer, marking a paradigm shift from the postoperative to preoperative chemo-radiation therapy approach. However, although the addition of 5-FU-based chemotherapy has reduced local recurrence, no benefit has been observed in the occurrence of distant metastases or overall survival, underlining the need for more effective systemic chemotherapy (Table 25.1). Moreover, according to the present knowledge, the benefit of postoperative chemotherapy after preoperative chemo-radiation therapy remains controversial. Indeed, the positive trend in improvement of overall survival reported in the EORTC 22921 trial suggested a possible benefit in some groups of patients at higher risk of recurrence, and further studies will be required to clarify this issue.

SELECTION OF CHEMOTHERAPY

5-Fluorouracil

For many years, 5-FU has been the single available agent to combine with pelvic radiation therapy for the treatment of locally advanced rectal cancer patients, on the grounds of its potent radiosensitizing properties, and its inhibition effect on thymidylate synthase. This enzyme plays a key role in DNA synthesis, and has been shown to have a prognostic value in locally advanced rectal cancer patients. Indeed, from an analysis of the data of the NSABP R01 trial, Johnston *et al*. (1994) have demonstrated that patients whose tumors contained high levels of thymidylate synthase had a worse clinical outcome when compared to patients whose tumors contained low levels of thymidylate synthase.

Although the efficacy of 5-FU in rectal cancer was recognized by the NCI Consensus Conference in 1990, its optimal schedule of delivery has not been established yet. Therefore, a main issue in the design of postoperative trials has been the identification of the optimal modality of 5-FU administration. The four-arm NCCTG 864751 trial reported by O'Connell *et al*. (1994) was designed to compare the delivery of intravenous 5-FU as a bolus or as a continuous infusion

during postoperative pelvic radiation therapy, and to assess the addition of semustine to 5-FU. In this study, all patients also received four cycles of bolus 5-FU, 5 days a week every 4 weeks, 2 cycles before and two cycles after chemo-radiation therapy. When compared with 5-FU bolus (500 mg/m^2 for 3 days in the first and fifth week of radiation therapy), patients who received 5-FU continuous infusion (225 mg/m^2/daily in the first and fifth week of radiation therapy) had a significant decrease in the overall recurrence rate (37% *vs.* 47%, P = .01), and an improvement in 4-year overall survival (60% *vs.* 70%, P = .005). Differences were also found in the acute toxicities. Patients receiving 5-FU continuous infusion had a significant increase of severe diarrhea (24% *vs.* 14%, P < .01), and a significant decrease of severe leukopenia (2% *vs.* 11%, P < .01), when compared to patients treated with bolus 5-FU. Semustine did not add any therapeutic benefit, therefore, it was no longer recommended. Based on these results, two cycles of bolus 5-FU before and after postoperative pelvic radiation therapy, and 5-FU continuous infusion during radiation therapy, was established as the standard adjuvant treatment in locally advanced rectal cancer. Two subsequent postoperative trials have evaluated the biochemical modulation of 5-FU with leucovorin and/or levamisole (INT 0114), and the incorporation of 5-FU continuous infusion throughout the entire adjuvant treatment (INT 0144). INT 0114 was a four arm trial, in which all patients received six cycles of postoperative 5-FU bolus, plus concurrent radiation therapy during cycles three and four, and it had the goal of determining whether modulated 5-FU (5-FU plus low dose leucovorin; 5-FU plus levamisole; 5-FU plus leucovorin and levamisole) was superior to 5-FU alone. The final results reported by Tepper *et al*. (2002) did not show any advantage for leucovorin or levamisole-containing regimens over 5-FU alone.

Following the positive results of 5-FU continuous infusion reported in the NCCTG 864751 trial, the three-arm INT 0144 trial was designed to determine whether there was a benefit for 5-FU delivered as continuous infusion throughout the entire six cycles of adjuvant chemotherapy as compared to 5-FU delivered as continuous infusion only during radiation therapy. A third arm, with a 5-FU bolus modulated by leucovorin and levamisole for the whole adjuvant treatment, was also adopted for practical concerns regarding the use of central venous catheters. The results recently reported by Smalley *et al*. (2006) showed that the administration of 5-FU continuous infusion throughout the whole adjuvant treatment did not improve disease free survival and overall survival compared with 5-FU delivered as continuous infusion only during radiation therapy. Furthermore, the bolus modulated 5-FU regimen produced results similar to those reported by the continuous infusion-based arms. Moreover, occurrence of toxic death (1%), and of severe gastrointestinal toxicity (about 40%) was similar in all arms, whereas grade 3–4 hematologic toxicity was much more common in the bolus arms (~ 50% *vs.* 4%). The results of INT 0144 provided evidence that there is no meaningful difference in efficacy between different modalities of delivery of 5-FU in the adjuvant treatment of rectal cancer, leaving the choice to physicians' preference and patients' compliance.

In the preoperative approach, the different schedules of 5-FU administration have thus far shown similar outcomes, although randomized trials addressing this issue have yet to be reported. Indeed, in the EORTC 22921 and FFCD 9203 trials, in which a 5-FU bolus regimen was adopted, the pathologic complete response, the local and distant recurrence, and the disease free survival and overall survival rates were similar to those reported in the German CAO/ARO/AIO 94 trial, in which 5-FU continuous infusion was utilized. However, in the last few years, a more effective downstaging (to increase the possibility of performing sphincter-preserving surgery), and a better control of distant spread have clearly emerged as compelling goals in the treatment of locally advanced rectal cancer. Therefore, recently available cytotoxic drugs and biologic agents were considered as excellent candidates for integration into a preoperative chemo-radiation therapy approach.

Oral Fluoropyrimidines

Capecitabine and uracil-tegafur are two pro-drugs of 5-FU that can be administered orally. They provide an attractive alternative to 5-FU because, when taken for several days, they mimic the pharmacokinetics of 5-FU continuous infusion, while avoiding the potential complications associated with central venous access. There is evidence that capecitabine administration allows for a more selective delivery of 5-FU, due to an enzymatic conversion at the cancer cell level, and its efficacy appears well documented both in adjuvant and first-line treatment of colorectal cancer. Therefore, several phase II trials have extensively evaluated the use of this drug in the preoperative chemo-radiation therapy approach. Kim *et al*. (2002) reported a 63% tumor downstaging, and a 31% pathologic complete response, with two cycles of an intermittent schedule of capecitabine (825 mg/m^2 twice daily) and leucovorin (20 mg/m^2/daily) for 14 days, followed by a 7-day rest, during radiation therapy for locally advanced rectal cancer patients. No grade ≥3 hematologic toxicity was reported, while severe diarrhea affected 4% of patients.

De Paoli *et al*. (2006) prospectively evaluated a continuous regimen of capecitabine given 825 mg/m^2 twice daily continuously during pelvic radiation therapy (50.4 Gy in 28 fractions) in patients with locally advanced rectal cancer. A downstaging was reported in 57% of patients, and a pathologic complete response in 24%. Treatment was well tolerated, with only six patients (11%) suffering from grade 3 toxicity. Das *et al*. (2006) have retrospectively compared the safety and efficacy of capecitabine delivered during pelvic radiation therapy in 89 patients with rectal cancer with those reported in a matched series of 89 patients previously treated with 5-FU continuous infusion, reporting a similar low occurrence of grade 3–4 toxicity, and comparable local and distant failure rates. Overall, capecitabine combined with pelvic radiation therapy has shown pathologic complete response rates comparable to those reported with 5-FU. An ongoing randomized phase III study (NSABP trial R04) is currently comparing, in a 2 × 2 factorial design, the combination of 5-FU continuous infusion *vs.* capecitabine, with or without oxaliplatin, during preoperative radiation therapy for locally advanced rectal cancer.

A limited amount of data is available on the use of uracil-tegafur (UFT) combined with pelvic radiation therapy. UFT is a mixed compound combining (in a fixed molar ratio of 4:1) tegafur, a prodrug of 5-FU, and uracil, an inhibitor of the main enzymatic catabolic pathway of 5-FU. In a multicenter phase II study, Fernandez-Martos *et al*. (2004) evaluated UFT given 400 mg/m^2 in three daily fractions (5 days a week, for 5 weeks) during pelvic radiation therapy (45 Gy in 25 fractions) in 94 locally advanced rectal cancer patients. Diarrhea was the most frequent grade ≥3 side effect of this treatment (14%), whereas only one patient had hematologic grade ≥3 toxicity. The downstaging rate was 54%, but a pathologic complete response was seen in only 9% of patients.

Raltitrexed

Raltitrexed is a quinazoline folate analogue with radiosensitising properties acting as a specific thymidilate synthase inhibitor. Since this drug displays a long half-life, it should provide a biological behavior similar to 5-FU continuous infusion. Gambacorta *et al*. (2004a) have treated 54 patients with T3 or T2N+ rectal cancer with raltitrexed 3.0 mg/m^2 (days 1, 19 and 38) and concurrent pelvic radiation therapy (50.4 Gy), reporting a total of 16.6% grade 3 toxicity of any type, and a very low occurrence of diarrhea (5.5% grade 1–2, none grade 3–4). At pathologic examination, 24% of patients showed a pathologic complete response, and 18.5% had only isolated residual cancer cells. These data suggested that raltitrexed, delivered in a more convenient schedule, yields similar results to 5-FU in a combined modality treatment for locally advanced rectal cancer.

Irinotecan

Irinotecan is suitable for its inclusion in a preoperative chemo-radiation therapy approach because of its radiosensitizing properties and efficacy in combination with 5-FU/leucovorin in the treatment of metastatic colorectal cancer, as reported by Douillard *et al*. (2000). Therefore, several studies have evaluated a preoperative chemo-radiation therapy regimen for locally advanced rectal cancer including irinotecan in combination with 5-FU or capecitabine.

Mehta *et al*. (2003) have evaluated the combination of 5-FU continuous infusion 200 mg/m^2/daily on days 1–33, and irinotecan 50 mg/m^2 weekly for 4 weeks, during preoperative pelvic radiation therapy (50.4 Gy) in 32 patients with T3N0-1 rectal cancer. This schedule caused a high incidence of grade 3 diarrhea (28%), although it was associated with an impressive 37% pathologic complete response rate. The addition of irinotecan to 5-FU continuous infusion during preoperative pelvic radiation therapy for locally advanced rectal cancer has been also assessed by the Radiation Therapy Oncology Group (RTOG) trial 0012. In this phase II study, reported by Mohiuddin *et al*. (2006), 106 patients randomly received either hyperfractionated *bid* pelvic radiation therapy (total dose, 45.6 Gy plus a boost of 9.6 Gy) with 5-FU continuous infusion (225 mg/m^2/daily), or a single daily fraction of pelvic radiation therapy (total dose, 45 Gy plus a boost of 5.4 Gy) with 5-FU continuous infusion (225 mg/m^2/daily continuous infusion for

5 days a week) and irinotecan (50 mg/m^2 weekly × 4 weeks). The same proportion of pathologic complete response (26%) was reported in both arms, and no substantial difference in occurrence and/or severity of toxicity was reported. A more feasible regimen was assessed by Glynne-Jones *et al*. (2007), who conducted a dose finding study of irinotecan delivered on days 1–5 and 29–33 added to 5-FU continuous infusion (350 mg/m^2/daily) and leucovorin 20 (mg/m^2/daily) during pelvic radiation therapy (45 Gy in 25 fractions) in borderline/unresectable locally advanced rectal cancer patients. They recommended a daily dose of 18 mg/m^2 for irinotecan in the 5-day schedule; 6 of 20 (30%) patients receiving this dose level achieved a pathologic complete response. Toxicity profile and compliance were good, with 93% and 89% of patients completing radiation therapy and chemotherapy, respectively.

Other investigators have assessed the combination of weekly irinotecan with capecitabine during three-dimensional conformal pelvic radiation therapy (50.4 Gy). In a phase I/II trial reported by Klautke *et al*. (2006), the recommended doses were 750 mg/m^2 twice daily for capecitabine, and 40 mg/m^2 weekly × 6 weeks for irinotecan. Also in this study, grade 3 diarrhea was the most common toxicity observed in 37% of patients, and a pathologic complete response was reported in 19% of patients. Therefore, Willeke *et al*. (2007) evaluated in a phase II study capecitabine 500 mg/m^2 twice daily for 38 days plus irinotecan 50 mg/m^2 weekly for five doses during pelvic radiation therapy in 36 patients with locally advanced rectal cancer. Gastrointestinal adverse events were frequently observed, but a grade 3 diarrhea occurred in only 11% of patients. However, only 14% of subjects showed pathologic complete response (Table 25.2).

Oxaliplatin

Oxaliplatin is a reasonable candidate for a combined modality treatment, because it has shown radiosensitizing properties and confers clinical benefit in the treatment of metastatic colorectal cancer. Furthermore, the addition of oxaliplatin to 5-FU/leucovorin has been demonstrated by Andrè *et al*. (2004) to significantly improve the disease free survival of patients with surgically resected stage II and III colon cancer in comparison with 5-FU/leucovorin alone. These findings have prompted the implementation of several phase II trials of combined modality approach including oxaliplatin for locally advanced rectal cancer.

Gérard *et al*. (2003) have reported on the administration of oxaliplatin 130 mg/m^2 plus a 5-day continuous infusion 5-FU 350 mg/m^2/daily and 6 S-leucovorin 100 mg/m^2/daily for two cycles on the first and fifth week of pelvic radiation therapy (50 Gy) in 40 patients. This regimen was well tolerated, and no residual tumor was seen in 15% of patients.

A dose finding study of weekly oxaliplatin in addition to 5-FU continuous infusion (225 mg/m^2/daily) during pelvic radiation therapy was reported by Aschele *et al*. (2005). They recommended a dose for oxaliplatin of 60 mg/m^2, and treated 25 patients with this regimen, reporting a 28% pathologic complete response, and grade 3 diarrhea in only 16% of subjects. Two ongoing randomized phase III studies (NSABP R04 and STAR trials) are currently evaluating the combination of weekly oxaliplatin with 5-FU continuous infusion and preoperative radiation therapy for locally advanced rectal cancer.

TABLE 25.2. Main phase II trials of preoperative chemoradiotherapy with irinotecam.

Author (no. patients)	Clinical tumor staging	Regimen	RT dose (Gy)	Main toxicity, grade ≥ 3 (%)	pCR (%)
Metha (32)	T2-3/N0-1: transrectal ultrasound	Cl 5FU 200 mg/m^2, days 1–33 CPT-11 50 mg/m^2, weekly × 4	50.4	Diarrhoea (28)	37
Mohiuddin (106)	T3-4: clinical staging	Cl 5FU 225 mg/m^2, 7 days a week or Cl 5FU 225 mg/m^2, 5 days a week CPT-11 50 mg/m^2, weekly × 4	*55.2 for T3 *60 for T4 or 50.4 for T3; 54 for T4	Gastrointestinal (35)	26
Glynne-Jones (20)	Borderline or unresectable T3-4: clinical staging or CT or MRI	Cl 5FU 350 mg/m^2 + LV 20 mg/m^2, days 1–5, 29–33 CPT-11 18 mg/m^2, days 1–5, 29–33	45	Diarrhoea (5) and Neutropenia (5)	30
Klautke (16)	T2-4/N0-1: transrectal ultrasound	Capecitabine 750 mg/m^2 b.i.d., days 1–43 CPT-11 40 mg/m^2, weekly × 6	50.4 + 5.4	Diarrhoea (37)	19
Willeke (36)	T3-4 and/or N+: transrectal ultrasound	Capecitabine 500 mg/m^2 b.i.d., days 1–38 CPT-11 40 mg/m^2, weekly × 6	50.4	Leukocytopenia (25)	14

CT, computed tomograph; MRI, magnetic resonance imaging; RT, radiotherapy; pCR pathological complete response; Cl, continuous infusion; 5FU, 5-fluorouracil; CPT-11, irinotecan.
*Hyperfracionated pelvic RT, 1.2 Gy bid, was used.

The combination of oxaliplatin and capecitabine, delivered during preoperative pelvic radiation therapy for locally advanced rectal cancer, has also been explored. Rodel *et al*. (2003) conducted a phase I/II study to define the optimal oxaliplatin dose to deliver on days 1 and 8 of a 2-week oral administration of capecitabine (825 mg/m^2/twice daily) for two cycles during pelvic radiation therapy (50.4 Gy). The recommended dosage for oxaliplatin was 50 mg/m^2, and 26 patients were treated with this regimen: a 19% pathologic complete response was observed, and severe diarrhea occurred in only 8% of cases. Different results were reported by Machiels *et al*. (2005), who treated 40 patients with oxaliplatin 50 mg/m^2 weekly for 5 weeks plus capecitabine 825 mg/m^2 twice daily during pelvic radiation

therapy (total dose, 45 Gy); a pathologic complete response was obtained in 14% of patients, whereas severe diarrhea occurred in 30% of them. Glynne-Jones *et al*. (2006) reported that the recommended doses for continuous administration of capecitabine was 650 mg/m^2 twice daily in addition to two doses of oxaliplatin 130 mg/m^2, 4 weeks apart, during pelvic radiation therapy (45 Gy in 5 weeks) before surgery. In a phase II study, they treated 85 patients, but post-treatment pathology was available in 83 operated cases, and a pathologic complete response was achieved in 16 (19%) of them. Treatment was well tolerated, with only 9% of grade ≥3 diarrhea. A large phase III pan-European trial (PETACC-6) comparing capecitabine and oxaliplatin with capecitabine alone during chemoradiation is ongoing.

Gambacorta *et al*. (2004b) have reported interesting results on the association of oxaliplatin and raltitrexed during pelvic radiation therapy (50.4 Gy). In this phase I-II study they concluded that the recommended dose for oxaliplatin was 130 mg/m^2 in addition to raltitrexed 3.0 mg/m^2 administered on days 1, 19 and 38. Using this regimen in 30 patients with limited disease extent (T3N0/+), a good toxicity profile was found, with no grade ≥3 diarrhea, and a pathologic complete response rate of 30% was reported.

On the ground of preclinical and clinical findings, highlighting a synergistic cytotoxicity, and a positive pharmacokinetic interaction, for the sequential exposure to raltitrexed and 5-FU, a phase I-II study has been conducted by Avallone *et al*. (2006) to assess the addition of oxaliplatin and raltitrexed to a biweekly regimen of 5-FU/leucovorin given for three cycles during pelvic radiation therapy (45 Gy) in patients with poor risk locally advanced rectal cancer (T4, N positive, and T3N0 located ≤5 cm from the anal verge and/or circumferential resection margin ≤5 mm evaluated by MRI). The recommended doses were 100 mg/m^2 for oxaliplatin and 2.5 mg/m^2 for raltitrexed on day 1,900 mg/m^2 for 5-FU bolus and 250 mg/m^2 for leucovorin on day 2. This regimen yielded an impressive activity: a 42% pathologic complete response rate was observed in 31 treated patients. Main severe toxicities were neutropenia and diarrhea, occurring in 38% and 19% of patients, respectively (Table 25.3). Interestingly, Avallone *et al*. (2007) have recently reported that a slight reduction of 5-FU dose (800 mg/m^2) appeared to improve the safety of this combination (grade 3 diarrhea was seen in only 6% of patients), while retaining its activity (pathologic complete response rate, 50%). Moreover, after a median follow-up of 32 (range 4–50) months, they reported a 30-month disease free survival of 95%, with all patients achieving a pathologic complete response being recurrence-free.

Cetuximab

Cetuximab is a monoclonal antibody raised against the epidermal growth factor receptor (EGFR), whose overexpression is associated with resistance to radiation therapy. Moreover, cetuximab has demonstrated to be effective in the treatment of metastatic colorectal cancer patients both as a single agent and in combination with irinotecan, as reported by Cunningham *et al*. (2004). Therefore, a strong rationale exists for combining cetuximab with preoperative chemo-radiation therapy for rectal cancer.

Machiels *et al*. (2007) have reported in a phase I-II study that the addition of weekly cetuximab (initial dose 400 mg/m^2 given 1 week before the beginning of radiation

TABLE 25.3. Main phase II trials of preoperative chemoradiotherapy with oxaliplatin.

Author (n. patients)	Clinical tumor stage	Regimen	RT dose (Gy)	Main toxicity, grade ≥ 3 (%)	pCR (%)
Gerard (40)	T3-4 : transrectal ultrasound	Cl 5FU 350 mg/m^2 + LFA 100 mg/m^2 days 1–5 and 29–33 Oxa 130 mg/m^2, days 1 and 29	50.4	Diarrhoea (8)	15
Aschele (25)	T3-4 and/or N+: transrectal ultrasound	Cl 5FU 225 mg/m^2/day for 6 weeks OXA 60 mg/m^2, weekly × 6	50.4	Diarrhoea (16)	28
Redel (26)	Low-lying T2 seeking sphincter preservation or T3-4: transrectal ultrasound	Capecitabine 825 mg/m^2 b.i.d., days 1–14 and 22–35 OXA 50 mg/m^2, days 1,8,22 and 29	50.4	Diarrhoea (8)	19
Machiels (40)	T3-4 and/orN+: transrectal ultrasound	Capecitabine 825 mg/m^2 b.i.d., 5 days a week × 5 OXA 50 mg/m^2, weekly × 5	45	Diarrhoea (14)	30
Glynne-Jones (83)	Borderline or unresectable T3-4: clinical staging or MRI	Capecitabine 650 mg/m^2 b.i.d., 7 days a week × 5 Oxa 130 mg/m^2, days 1 and 29	45	Diarrhoea (9)	19
Gambacorta (30)	T3 and/or N+: transrectal ultrasound	RTX 3 mg/m^2 days 1, 19 and 38 Oxa 130 mg/m^2, days 1, 19 and 38	50.4	Leukocytopenia (10)	30
Avallone (31)	T4, N+ and T3N0 with CRM ≤5 mm and/or ≤5 cm anal verge: transrectal ultrasound and MRI	Oxa 130 mg/m^2 + RTX 2.5 mg/m^2 days 1, 15 and 29 5FU 900 mg/m^2 + LV 250 mg/m^2 days 2, 16 and 30	45	Neutropenia (38)	42

MRI, magnetic resonance imaging; RT, radiotherapy; pCR pathological complete response; Cl, continous infusion; 5FU, 5fluorouracil; OXA, oxaliplatin; RTX, raltitrexed.

therapy, followed by 250 mg/m^2 weekly for 5 weeks) to capecitabine 825 mg/m^2 twice daily given continuously during pelvic radiation therapy (45 Gy in 28 fractions) was feasible, with grade 3 diarrhea occurring in 15% of patients. However, a pathologic complete response rate of 5% was reported. Moreover, Hofheinz *et al*. (2006) have confirmed that the addition of weekly cetuximab to capecitabine

500 mg/m^2 twice daily and weekly irinotecan 40 mg/m^2 weekly for 5 weeks during pelvic radiation therapy was feasible and well tolerated. Furthermore, cetuximab was safely added also to a combination of oxaliplatin 50 mg/m^2 delivered on days 1 and 8 of a 2-week schedule of capecitabine (825 mg/m^2/twice daily) for two cycles during pelvic radiation therapy (50.4 Gy), as recently reported by Hipp *et al*. (2007) in a phase I-II study. However, this regimen delivered to 38 patients with T3–4 and/or N+ rectal cancer produced a surprisingly low rate of pathologic complete response (8%) when compared to that previously reported by the same group with the regimen of oxaliplatin and capecitabine without cetuximab (Rodel *et al*., 2003). In conclusion, additional preclinical and clinical studies are needed to better identify potentially sensitive patients, and to better define the activity of this biological agent in the chemo-radiation therapy treatment of rectal cancer.

Bevacizumab

Bevacizumab is a monoclonal antibody that binds to the vascular endothelial growth factor (VEGF). This antibody, when combined with chemotherapy in the first and second-line treatment of metastatic colorectal cancer patients, has been demonstrated to prolong the overall survival as reported by Hurwitz *et al*. (2004) and Giantonic *et al*. (2007). Moreover, an anti-VEGF treatment is one of the most promising approaches to increase the efficacy of radiation therapy. Therefore, the combination of bevacizumab with chemo-radiation therapy is intriguing in the treatment of locally advanced rectal cancer. Willett *et al*. (2005) in a phase I-II study found that the recommended dose for biweekly bevacizumab in addition to 5FU (225 mg/m^2/day continuous infusion during pelvic radiation therapy) was 5 mg/kg. Moreover, administering bevacizumab 2 weeks before chemo-radiation therapy, they showed that the tumor perfusion, the microvascular density, and the interstitial fluid pressure were substantially decreased as early as 12 days after the start of the anti-VEGF treatment. A phase II study is ongoing, and preliminary results show an encouraging tumor downstaging. Other phase II studies are evaluating bevacizumab in combination with capecitabine, with or without oxaliplatin, and radiation therapy in patients with locally advanced rectal cancer.

Altogether, these reported studies showed that a treatment intensification, combining different cytotoxic agents during preoperative pelvic radiation therapy, led to a constant increase of pathological responses, with a higher rate of pathologic complete responses. However, the flaw of these results is due to the heterogeneity of locally advanced rectal cancer patients included in these series, and to the lack of standardized clinical staging and pathologic assessment, which might have contributed to the reported high pathologic complete response rates. Moreover, a pathologic complete response after preoperative chemo-radiation therapy has not been validated yet as a surrogate end-point for long-term clinical outcome, while it remains to be defined whether an excellent pathologic response has an impact on the natural history of the disease, or it is merely associated with favorable characteristics of the patient and/or the tumor. Furthermore, few studies have reported long-term outcomes and late toxicity of combined treatment. Therefore, we still need further prospective data to confirm that an intensified treat-

ment of chemo-radiation therapy, achieving a higher rate of pathologic complete responses, will also positively affect the overall survival of patients. Ongoing clinical trials will provide some insight into this important issue.

PATIENT SELECTION

The common use of total mesorectal excision, and the shift from a postoperative to a preoperative chemo-radiation therapy approach, have shown that local recurrence appears to be less of a problem, whereas distant metastases remain the most common site of treatment failure in locally advanced rectal cancer patients. Therefore, a strategy for increasing the efficacy of chemotherapy should be pursued, but the price to be paid could be a higher risk of acute and late adverse effects. Moreover, locally advanced rectal cancers are a widely heterogeneous group of diseases, that may have a quite different prognostic implication. Therefore, a careful identification of patients at high risk of recurrence is a critical issue, because it is likely that not all patients with locally advanced rectal cancer need a primary and/or intensified approach.

Gunderson *et al*. (2004) conducted a pooled analysis on more than 3,700 patients with resectable rectal cancer, included in five randomized US trials performed before the introduction of total mesorectal excision, assessing the role of postoperative radiation therapy, chemotherapy or both, and reported widely different recurrence and overall survival rates according to both the T and N extension. Based on these parameters, they were able to identify four prognostic subgroups for recurrence: the low risk (T1-2/N0), the intermediate risk (T1-2/N1; T3/N0); the moderately-high risk (T1-2/N2; T3/N1; T4/N0); and the high risk group (T3/N2; T4/N1–2). Therefore, they advocated the use of a combined chemo-radiation therapy approach for patients with moderately-high or high risk tumors, because of their greater rates of both local and distant relapse. Conversely, the combined chemo-radiation therapy may represent an overtreatment for patients with an intermediate risk, in consideration of the low rate of local recurrence. For carefully selected patients of this group, surgery alone or surgery followed by postoperative chemotherapy could be more appropriate. A management with surgical resection alone could be considered for the low risk (stage I) patients, due to their low rate of both local and distant failure.

Although this was a retrospective and non comparative analysis, which included patients observed over a 13-year period, and treated with a variety of surgical techniques and treatment modalities, it was the first attempt at refining the selection of patients for different treatment strategies.

Doubts about the need for a combined chemo-radiation therapy approach for all patients with T3N0 rectal cancer were raised by the retrospective data reported by Willet *et al*. (1999). Indeed, they observed that patients with T3N0 tumors and more favorable characteristics (well or moderately differentiated histology, limited extension into the perirectal fat, and without lymphatic or vascular invasion) had an excellent outcome with surgery alone without adjuvant treatment. Conversely, Nissan *et al*. (2006) have recently shown that the lymphovascular invasion, and an elevated preoperative serum carcinoembryonic antigen (CEA) > 5 ng/mL,

were related with a higher incidence of pelvic recurrence and reduced survival in patients with early T3N0 tumors uniformly treated by surgery alone, suggesting that these selected patients could be candidates for additional therapy.

However, in the last few years the key role of the assessment of the circumferential resection margin for identifying patients with high risk of recurrence has clearly emerged, in addition to the T and N stage. Quirke *et al*. (1986) have shown that, rather than the proximal and distal margins, the involvement of the circumferential resection margin is critical for the outcome of locally advanced rectal cancer patients. Indeed, they demonstrated that local recurrence was greatly increased, and survival halved, for patients in which the distance between the deepest extent of the tumor and the radial margin measured ≤1 mm on microscopic examination. Furthermore, Nagtegaal *et al*. (2002a) analyzing the data of the Dutch trial, demonstrated that the circumferential resection margin involvement adversely affected the risk of local recurrence, even after optimal total mesorectal excision surgery. Moreover, they showed that a positive circumferential resection margin (≤1 mm) was also predictive for the development of distant metastases (37% *vs.* 15%), as well as of a lower 2-year survival (70% *vs.* 90%). Interestingly, data of this trial reported by Marijnen *et al*. (2003) have also demonstrated that radiation therapy alone cannot sufficiently compensate for a positive circumferential resection margin, supporting the need in these patients for preoperative chemo-radiation therapy to achieve greater tumor shrinkage and an R0 resection. Moreover, the poor prognosis of patients with low (less than 5 cm from the anal verge) rectal cancer has also been ascribed to the higher frequency of circumferential resection margin involvement occurring for the natural "coning-in" of the mesorectum in this anatomic site. Indeed, Nagtegaal *et al*. (2005) have demonstrated that the frequency of a positive circumferential resection margin was more than doubled in low rectal cancer compared to tumors located over 5 cm from the anal verge (26.5% *vs.* 12.6%, $P < .001$), and confirmed a significantly worse local recurrence and overall survival for patients with positive circumferential resection margins. These findings demonstrated the importance of the evaluation of circumferential resection margin involvement with diagnostic imaging. In the last years, magnetic resonance imaging with a phased-array coil has emerged as a highly accurate tool to predict circumferential resection margin involvement. Beets-Tan *et al*. (2001) have demonstrated that circumferential resection margin involvement can be predicted with a high confidence when this margin is less than 5 mm on preoperative magnetic resonance imaging. Interestingly, Burton *et al*. (2006) have demonstrated that a magnetic resonance imaging based strategy, intensifying the preoperative treatment in those patients with a potential involvement of the circumferential resection margin, resulted in a significant reduction of pathologically positive circumferential resection margin. Moreover, recent developments of new magnetic resonance imaging contrast agents, like Ultra Small Super Paramagnetic Iron Oxide (USPIO), have shown that magnetic resonance imaging may be very promising also for the detection of lymph node metastases. Therefore, with the increasing use of preoperative treatment, magnetic resonance imaging has

taken a key role in the staging of patients with locally advanced rectal cancer, because it allows more accurate staging, reducing the risk of overtreating patients with an early-stage tumor, and improving the identification of patients with high risk of recurrence.

Another critical point is the selection of patients for whom a risk-adapted adjuvant chemotherapy should be utilized, considering that not all tumors respond uniformly to the preoperative treatment, and taking also into account the poor adherence to postoperative chemotherapy. Indeed, recent results of the EORTC 22921 trial failed to demonstrate a significant impact of postoperative chemotherapy on survival, although a late difference seemed to emerge, at approximately 2 years for disease free survival, and at 4 years for overall survival. This trend indicated that certain subgroups of patients may benefit from adjuvant 5-FU-based chemotherapy. Moreover, one might speculate that, besides patients that do not require adjuvant chemotherapy, there may be patients needing more effective chemotherapy.

Rodel *et al*. (2005) have evaluated the prognostic significance of tumor regression in the cohort of 406 rectal cancer patients treated by preoperative chemo-radiation therapy in the CAO/ARO/AIO 94 trial. They reported that tumor regression grading could predict long-term outcome: a complete or intermediate pathological response after preoperative chemo-radiation therapy was related with an improved disease free survival. On the other hand, although in the series reported by Rodel tumor regression grade was proven to be prognostically valuable in the univariate analysis, the pathological T (ypT) and N (ypN) status was the most important independent factor affecting the disease free survival in the multivariate model. In particular, a positive ypN was the strongest prognostic factor for local and distant recurrence, and disease free survival. Fietkau *et al*. (2006) analyzed the results of 95 patients treated with preoperative 5-FU-based chemo-radiation therapy followed by R0 resection (and adjuvant 5-FU-based chemotherapy in 65 patients), observing that ypN status after chemo-radiation therapy was the only significant prognostic parameter. Interestingly, they found that the 3-year disease free survival for patients with ypN0 was excellent, independently of whether they received postoperative chemotherapy (87.5% *vs.* 87.7%), whereas patients with ypN2 status had poor 3-year disease free survival (30%) despite adjuvant chemotherapy.

On the whole, these retrospective data indicated that persistently metastatic nodes are predictive of an unfavorable prognosis, suggesting that postoperative chemotherapy should be intensified in these patients, while it could possibly be spared in patients with ypN0.

Pathologic circumferential resection margin involvement after preoperative chemo-radiation therapy was recently reported to have a negative prognostic impact on local recurrence and overall survival by Mawdsley *et al*. (2005). In their study, a total of 150 patients with borderline resectable or unresectable locally advanced rectal cancer were treated with preoperative long-course pelvic radiation therapy and 5-FU-based chemotherapy. A significant difference was found in both 3-year disease free survival (52% *vs.* 9%, $P < .001$) and overall survival (64% *vs.* 25%, $P < .0001$) between patients who had a pathologically negative circumferential

resection margin and those who did not, underlining that the circumferential resection margin after preoperative chemo-radiation therapy has a major prognostic role.

In the last years, a careful histological assessment of surgical specimens has been demonstrated to be a crucial point to obtain correct prognostic information. Several studies have underlined that accurate lymph node assessment is mandatory, particularly in tumors staged as T3N0. Tepper *et al*. (2001) have shown that the number of retrieved nodes impacted on the outcome of patients with pN0 status, resulting significantly associated with disease free survival and overall survival. Since it has been shown that primary pelvic radiation therapy reduces the number of retrieved lymph nodes, the increasing use of a preoperative combined approach could negatively affect the accuracy of N staging.

However, Avallone *et al*. (2006) have shown that accurate pathologic assessment is feasible (with a median of 29 retrieved nodes) even after intensified preoperative chemo-radiation therapy treatment. Moreover, a careful pathologic evaluation may provide additional prognostic information, allowing to assess the quality of surgery. Nagtegaal *et al*. (2002b) showed that there was a significant relationship between the quality of the resection and the amount of clearance of the tumor: an increased frequency of circumferential resection margin involvement was seen in tumors with an incompletely removed mesorectum by inadequate surgery. However, in patients with positive circumferential resection margin, an incomplete resection did not increase the recurrence rate, whereas in patients with negative circumferential resection margin, an incompletely removed mesorectum doubled the recurrence rate (from 15% to 29%, $P = .03$), and decreased the overall survival (from 91% to 77%, $P < .05$). Therefore, an accurate pathologic evaluation is a key determinant of prognosis, and it plays a critical role for the assessment of the preoperative treatment, facilitating interstudy comparisons.

Molecular markers (such as K-ras, thymidilate synthase and p53) have been correlated with the clinical outcome in rectal cancer with controversial results. However, an interesting new approach to predict tumor response and long term outcome has recently been reported. In 30 patients treated as part of the CAO/ARO/AIO 94 trial Ghadimi *et al*. (2005) showed that gene expression profiling, using a 54-gene set, correctly predicted pathologic response, with an 86% specificity and 78% sensitivity. Their results suggested that pre-therapeutic gene expression profiling might be a useful method of identifying patients who need an intensified preoperative chemo-radiation therapy regimen. Interestingly, a recent prospective study of sequential ^{18}F-FDG PET assessment of rectal cancer during preoperative chemo-radiation therapy has demonstrated the predictive value of this technique for pathological response: in 33 patients, Cascini *et al*. (2006) showed that an early reduction (> 50%) of standardized uptake value of ^{18}F-FDG on PET, detected 12 days after the beginning of treatment, predicted the pathological response with a 100% accuracy. These results are provocative, because an early identification of non-responder patients might prompt alternative treatment strategies, whereas the prediction of a good pathologic response could allow to plan a more conservative surgical approach. However, these

new intriguing results require validation in large prospective studies.

In summary, there is compelling evidence that locally advanced rectal cancers are a large and prognostically heterogeneous group of diseases, in which circumferential resection margin involvement and the location of the tumor, besides the T and N extent, are also critical factors in defining the risk of recurrence. Therefore, a more accurate staging, and a stratification by risk factors should be adopted in clinical trials. Moreover, opposite risk-adapted strategies should be investigated, reserving initial surgery to patients with low risk, and a more intense chemotherapy, included in a combined preoperative approach, for those at high risk of recurrence. Some subsets of rectal cancer patients could benefit from adjuvant chemotherapy or even its intensification after preoperative chemoradiation therapy. However, only a careful pathologic assessment may provide reliable data concerning patient prognosis, and it is crucial to draw meaningful conclusions from clinical trials (Table 25.4).

TABLE 25.4. Summary of pretreatment and postreatment risk factor predictive of recurrence in locally advanced rectal cancer patients.

Clinical risk factors before preoperative treatment	Pathological risks factor after preoperative treatment
	ypN status
T and N status	ypCRM
CRM involvement	TRG
Distance from anal verge	Number of nodes retrieved in ypN0
Values of CEA	Quality of surgery

T, tumor; N, lymph node; CRM, circumferential resection margin; yp, pathologic post-treatment status; TRG, tumor regression grade.

CONCLUSIONS

In conclusion, in the last few years the common use of total mesorectal excision, and the shift from a postoperative to a preoperative chemo-radiotherapy approach have substantially reduced the risk of local recurrence in locally advanced rectal cancer.

Chemotherapy has been shown to play a relevant role in the management of locally advanced rectal cancer, but the integration of novel cytotoxic drugs and biologic agents in combined therapy is needed, in an attempt to improve the efficacy of downstaging and the control of distant spread. However, the key to further improvement in the clinical management of locally advanced rectal cancer will be accurate selection of patients, based on clinic-pathologic features and molecular and genetic markers, for whom different risk-adapted strategies of treatment should be adopted. Moreover, early prediction of pathological tumor response by genomic approaches and imaging modalities, such as DNA microarrays and ^{18}F-FDG PET, could lead to further tailoring of patient management. Refinements of multimodal therapy in order to maximize the potential of cure and minimize the impact on the quality of life of locally advanced rectal cancer patients, will only derive from an integrated approach of a highly skilled multidisciplinary team.

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26
Colorectal Cancer Liver Metastases: Neoadjuvant Therapy with Bevacizumab

Thomas Gruenberger and Birgit Gruenberger

INTRODUCTION

Metastatic colorectal cancer (mCRC) is still thought to be a disease resulting in early death in most cases, and patients are depressed by the diagnosis and its dismal prognosis described to them by their physicians. Multidisciplinary teams have, however, considerably changed the survival options, and the combination of effective chemotherapy plus targeted agents and surgery are nowadays leading to the potential cure for some mCRC patients. This highly specialized approach to the treatment of mCRC needs to be distributed to patients, physicians and health care providers to maximize the potential benefit (Gruenberger *et al*., 2004).

Advances in the outcome of mCRC patients have been achieved by both medical and surgical oncologists, but the most important step was the combination of the knowledge of these specialists in tumor treatment boards where experienced radiologist and radiation therapists were included in a structured algorithm to treat mCRC patients.

Inclusion of targeted agents into the neoadjuvant (if potentially resectable) or palliative (primarily unresectable) treatment plan has resulted in unexpected response rates. Thereby, surgical oncologists are confronted with smaller tumors (Figure 26.1). Therefore, liver surgery is the treatment for remaining metastases, but the problem of chemotherapy-induced liver alteration exists (Figure 26.2, Rubbia-Brandt *et al*., 2004).

These advances led to a change of the primary intention to treat mCRC for the purpose of prolonging symptom control and survival for a reasonable time frame of 1–2 years to the purpose of curing mCRC patients. The major unanswered questions are which treatment combination will lead to the highest response rates, for how long should we treat to postpone hepatic changes, how long do we have to wait after antiangiogenic therapy prior to surgery, should we give adjuvant therapy and if for how long? Some of these questions are still unanswered. Some data especially regarding Bevacizumab are available and are described below.

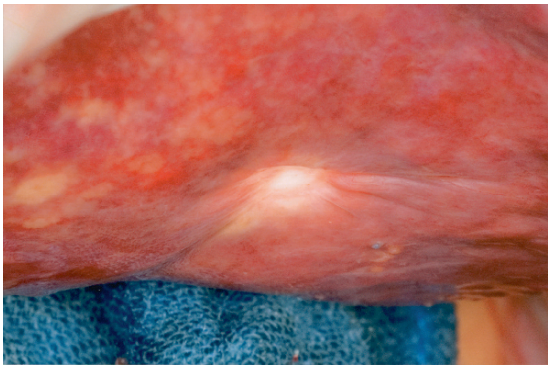


FIGURE 26.1. Remaining colorectal cancer liver metastasis after 4 months of palliative treatment

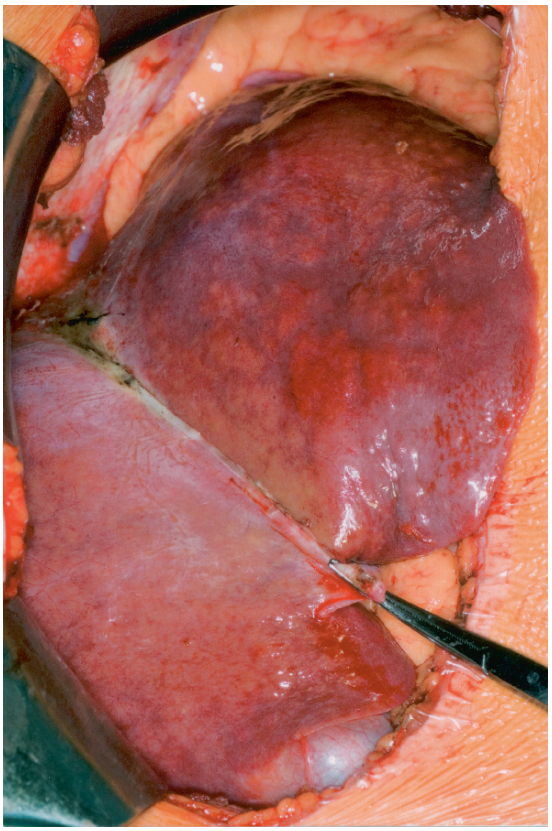


FIGURE 26.2. Severely altered liver after 4 months of Oxaliplatin containing therapy in a young fit lady with a normal body mass index of 23

VALUE OF BEVACIZUMAB IN THE NEOADJUVANT SETTING

Efforts to inhibit angiogenesis to control the growth and spread of cancer cells began more than 30 years ago (Folkman *et al*., 1971). Colorectal cancer is one of the best studied models of tumor angiogenesis, and numerous angiogenic growth factors have been identified. These include vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor (PD-ECGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs), angiogenin, thrombospondin, angiopoeitins, and integrins (Wray *et al*., 2004). The most successful antiangiogenic strategy to date has focused on neutralizing VEGF, a soluble glycoprotein that is an important regulator of physiological and pathological angiogenesis (Ferrara *et al*., 2003). There are two VEGF receptor tyrosine kinases, VEGFR-1 and VEGF-2, and the latter appears to be the major mediator of the angiogenic effects of VEGF.

Bevacizumab (Avastin®) is a humanized antibody directed against the VEGF-1 and VEGF-2 ligands. Preclinical studies demonstrated that Bevacizumab binds to and neutralizes all human VEGF-A isoforms. In addition to its direct antiangiogenic effects, Bevacizumab may also improve the delivery of chemotherapy by reducing interstitial pressure in tumors (Jain, 2001).

Combining antiangiogenic therapy with chemotherapy has become the standard of care for the first line treatment of mCRC patients since the pivotal trial by Hurwitz *et al*. (2004) who demonstrated a

significant prolongation of response rates (RR), progression-free survival (PFS) and overall survival (OS) for a combination of bolus fluorouracil and irinotecan (IFL) plus Bevacizumab compared to IFL alone. Bevacizumab has recently also proven its efficacy in extending PFS in the combination with FOLFOX/XELOX presented by Saltz *et al*. (2007a), and acts as the only targeted agent currently approved in the first line setting.

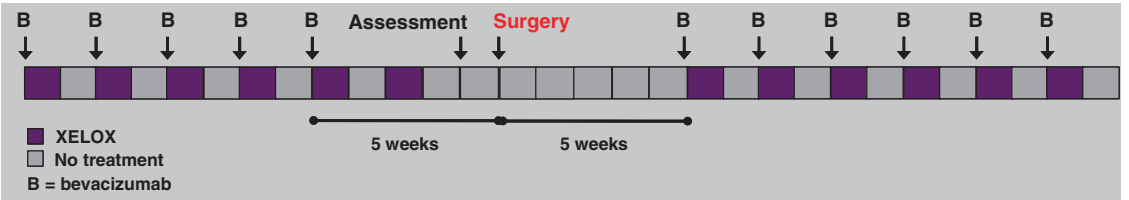
SURGERY AFTER BEVACIZUMAB IN THE NEOADJUVANT SETTING

The curative intent in mCRC patients after the treatment with Bevacizumab has not been explored prospectively due to its potential side effects in regards to wound healing, intra-operative bleeding, and liver regeneration (Chong *et al*., 2005). Unfortunately, there are no strong clinical data to guide recommendations on the timing of hepatic resection following neoadjuvant therapy with bevacizumab. The current recommendation call for an interval of 6–8 weeks after the last dose due to the half-life of the drug (Ellis *et al*., 2005). It is also recommended to administer another course of the cytotoxic regimen during the waiting period to prevent tumor regrowth.

Our group has carried out a prospective pilot trial in which we performed potentially curative liver resection 5 weeks after the last administration of bevacizumab. Inclusion criteria were a high risk of early disease recurrence in resectable patients (Fong *et al*., 1999). Patients were treated with a combination of oxaliplatin (85 mg/m^2 day 1) and capecitabine (3,500 mg/m^2 day 1–7) together with bevacizumab (5 mg/kg day 1) every 2 weeks for five cycles, and a last cycle without bevacizumab for a total of 3 months. If restaging demonstrated partial response or stable disease, liver resection was performed 5 weeks after the last bevacizumab, 3 weeks after last oxaliplatin and 2 weeks after last capecitabine. Adjuvant treatment with the same regimen was started for additional 3 months 5 weeks after surgery, when wounds had completely healed (Table 26.1). Primary end points in this single institutional trial were feasibility, morbidity, and mortality; secondary endpoints were response rate and resectability rate. The trial recruited 56 patients and results of both primary and secondary endpoints for all patients were presented at the ASCO 2007 meeting (Gruenberger *et al*., 2007). Interim analyses were published earlier by Gruenberger *et al*. (2006a).

We were able to demonstrate that this combination achieves a high response rate (> 70%) and disease control rate (> 90%)

TABLE 26.1. Neoadjuvant treatment schedule Xelox (Xeloda®, Oxaliplatin) + bevacizumab prior surgery.



and curative resectability rate was 95%. Patients demonstrating with progressive disease at routine staging after 3 months treatment were offered second line treatment because curative surgery in these patients, even if possible, does not prevent early recurrence (within 6 months) as demonstrated by Adam *et al*. (2004). We did not experience increased intraoperative bleeding and required perioperative blood transfusions in < 10% of all resections, despite the fact that major liver surgery (removal of ≥ 3 segments) was performed in > 35% of all patients. Perioperative morbidity was 21%, and was not directly linked to the administration of Bevacizumab; a single wound infection was noticed in a patient who underwent synchronous liver and bowel resection; we did not observe a single postoperative death. Dose reduction was required in one third of the enrolled patients and the most common side effect was diarrhea, followed by polyneuropathy (PNP) and neutropenia. Postoperative liver regeneration was normal in all but one patient (steatohepatitis revealed in histology), which was assessed in postoperative liver function tests and three monthly computed tomography (CT) scans. Translational research data from our group revealed a similar postoperative VEGF serum level increase after 5 weeks without bevacizumab as in patients without neoadjuvant bevacizumab treatment (Brostjan, 2008), which supports the clinical experience of normal recovery of liver function in almost all patients.

Although not strictly performed after a defined period of time following the last bevacizumab dose, other groups have also demonstrated that surgery can be performed safely and without risk of perioperative or long-term risk of increased complications. A single-institution retrospective study presented by Kesmodel *et al*. (2007) of 125 patients who underwent hepatic surgery showed that neoadjuvant bevacizumab plus chemotherapy did not increase the rate of any complications (49% versus 43%, $P = .51$), hepatobiliary (5% versus 11%, $P = .20$) or wound-healing complications (28% versus 25%, $P = .68$) compared with neoadjuvant chemotherapy alone. Recent data from NO16966 showed that Bevacizumab in combination with XELOX or FOLFOX allowed surgery with curative intent in 59/699 (6.1%, intent-to-treat population) and 34/177 (19.2%, liver metastases only) patients as presented by Saltz *et al*. (2007b) without increased postoperative morbidity.

FUTURE PROSPECTIVE

Surgical oncologists and hepatobiliary surgeons conclude that it is not the targeted agent that interferes with perioperative complications but the length of combination chemotherapy given, as outlined by Aloia *et al*. (2006). Therefore, care should be given to reduce the chemotherapy cycles prior to surgery by utilizing the most effective therapies to induce possible major response after a limited time on treatment. The best regimen to achieve this effort is still to be defined and mandates that we continue to enrol patients in well-designed clinical trials.

If prolonged treatment (> 3 months) is necessary to achieve potential resectability special attention should be given to the following risk factors of postoperative liver dysfunction: necessity of an extended resection, obese patients, and preoperative impaired liver function.

A combination of such risk factors makes the induction of a larger future remnant liver (the liver remaining after liver resection) obligatory (e.g., portal vein embolization, Gruenberger *et al*., 2006b).

CONCLUSION

Treatment approach to metastatic colorectal cancer patients has recently changed considerably due to exciting results achieved with the inclusion of targeted agents in the management algorithm. Progression-free and overall survival figures have been substantially extended, and the secondary resectability rate for cure has become an endpoint in recently designed trials. Nevertheless, attention should be attributed to a number of essential points: (1) a tumor board (including at least a medical- and surgical oncologist and a radiologist) should decide on treatment plan and review patient scans at regular intervals (e.g., 3 monthly); (2) the most effective treatment combinations should be used in the first line setting to reduce the number of cycles prior to a potential curative approach; (3) complex surgical procedures for liver metastases should by referred to a specialized hepatobiliary centre to avoid postoperative morbidity and mortality; (4) centres with specific interest in mCRC and participation in trials evaluating the best treatment combination should be preferred for referral.

In our study, bevacizumab did not increase the surgical morbidity if potential curative liver surgery was performed electively after 5 weeks without Bevacizumab. Our clinical and experimental data did not note an increased peri- or intraoperative bleeding risk nor was wound healing a problem. Discussions regarding impaired liver regeneration cannot be supported based on our experience, as we have not seen perioperative liver dysfunction or regeneration problems in the follow-up period even after adjuvant treatment, including bevacizumab, in the vast majority of our patients.

The best chemotherapy combined with targeted agent is currently being evaluated in large multicentre phase II and III trials, and special emphasis is given to the percentage of patients becoming secondary resectable for cure in the exciting area of metastatic colorectal cancer treatment.

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27
Colorectal Liver Metastases: Radiofrequency Ablation

Andrew McKay, Elijah Dixon, and Oliver Bathe

INTRODUCTION

In North America, colorectal cancer (CRC) is the fourth most commonly diagnosed cancer and the second leading cause of cancer death (American Cancer Society, 2005). Nearly half of patients will develop liver metastases at some point during the course of their disease, with 15–25% having metastases at the time of diagnosis. Even with recent dramatic improvements in systemic therapies for metastatic CRC, long-term survival without hepatic resection is extremely rare. Unfortunately only a small fraction of patients with hepatic metastases are candidates for curative resection. Patients with chronic liver disease may not be candidates for hepatic resection because this might lead to hepatic insufficiency from inadequate liver reserve. Patients with prolonged exposure to preoperative chemotherapy can also predispose patients to developing postoperative liver failure after extensive resections (Karoui *et al*., 2006). Even in patients with normal preoperative hepatic function, curative resection of multiple lesions can lead to inadequate hepatic reserve. To allow technical resectability in a patient with normal hepatic parenchyma, at least 25% of the original liver volume or at least two segments must remain (Mullin *et al*., 2005). The liver remnant must be larger for patients with hepatic dysfunction. Other patients may not be surgical candidates because the proximity of liver lesions to critical vascular or biliary structures can make resection technically difficult.

Because most people will not be candidates for hepatic resection, several local ablative techniques have been developed to provide aggressive local treatment to a broader range of patients. These ablative treatments have been designed to destroy tumor cells while sparing uninvolved hepatic parenchyma. One of the first such alternative procedures studied was percutaneous ethanol injection (PEI). This procedure has been shown to be an effective treatment for nodular-type hepatocellular carcinoma (Livraghi *et al*., 1995), but there are limitations to this technique in patients with colorectal liver metastases. The alcohol tends to preferentially spread in the adjacent normal liver parenchyma rather than the hard tumor, which limits its ability to completely destroy the entire tumor (Amin *et al*., 1993). Complications of the procedure include biliary reflux with secondary sclerosing

cholangitis. Cryoablation using liquid nitrogen has also been used. Its complications are significant, and include cracking of the frozen liver, severe hemorrhage, cold injury to adjacent organs, biliary fistulae, coagulopathy, thrombocytopenia, myoglobinuria, renal failure, hepatic abscess, and pleural effusions (Curley, 2001).

Radiofrequency ablation (RFA) has been developed with the hope of avoiding the drawbacks of other ablative techniques such as PEI and cryoablation. In the context of CRC, the aim is to increase the number of patients with liver metastases that might benefit from liver-directed treatment with curative intent. The ultimate goal is to achieve a similar survival as can be achieved with hepatic resection, but with less morbidity and impact on quality of life. The purpose of this chapter is to provide an overview of the current status of RFA in the treatment of colorectal liver metastases.

TECHNICAL ASPECTS OF RADIOFREQUENCY ABLATION

The use of radiofrequency energy for medical applications has been described as early as the late 19th century by the French physicist, Jaques-Arsène d'Arsonval. The principles have changed little since the first electrocautery machine was developed by Bovie and Cushing in the 1920s. Radiofrequency ablation devices are very similar to commonly used electrocautery units, in that they both use radiofrequency energy. The difference is that rather than using a brief, focused burst of radiofrequency energy for a bloodless incision or to coagulate bleeding vessels, RFA devices employ a more dispersed distribution of relatively milder radiofrequency energy to produce a more extensive sphere of tissue ablation (Ni *et al*., 2005).

A typical RFA device consists of a current generator and an electrode. Several large conductive grounding pads are applied to the patient's skin, and the radiofrequency generator produces a radiofrequency voltage between the radiofrequency electrode and the conductive grounding pad through the patient's body. This radiofrequency energy is an alternating current that generally has a frequency ~ 500 kHz (i.e., between audio and infrared frequencies). The frequency is high enough that it can pass through the body without producing a neuromuscular response. At the site of the radiofrequency electrode, the current is emitted from a very small surface area, which results in a high current density and high electrical resistance around the tip of the electrode (Ni *et al*., 2005). Heat is then generated and concentrated in the tissue in the immediate vicinity of the electrode when a high frequency alternating current moves from the tip of the electrode into the surrounding tissue. The alternating current causes agitation of ions in the tissue as they move with the rapidly alternating current, resulting in the generation of frictional heat. At the other end of the circuit, the large surface area of the conductive grounding pad results in a low current density and a low electrical resistance, which prevents heat generation. The delivered current is inversely proportional to the square of the distance from the electrode, so the temperature falls rapidly as the distance from the electrode increases.

As tissue temperature increases, the likelihood that cells will undergo coagulation necrosis rises. When subjected to a temperature of 46°C for 60 min,

irreversible cell damage occurs (Larson *et al*., 1996). As the temperature increases further, the time necessary to produce this damage becomes less and less. At 60°C, cell death becomes inevitable (Thomsen, 1991). A typical RFA treatment can result in local tissue temperatures above 100°C (Curley, 2001). The histologic appearance of coagulative necrosis does not occur immediately, but takes several days to develop (Goldberg *et al*., 2000).

In order to destroy any microscopic foci of malignancy that may lie at the periphery of the tumor and to provide a margin for error, a 1 cm zone of surrounding normal liver parenchyma is usually included in the ablation field. In the past, this was problematic because a major limitation of RFA was the small area of necrosis that could be achieved. This increased the number of electrode deployments that were necessary to treat all but the smallest lesions. Several modifications to the electrodes have been designed to overcome this problem and to allow treatment of larger lesions (Ni *et al*., 2005). Some manufacturers designed cooled-tip electrodes that use chilled saline to cool the needle tip (Valleylab; Boulder, Colorado, USA, Invatec [Roncadelle, Italy], and Celon [Teltow, Germany]). This limits charring at the tip of the electrode that can restrict propagation of the radiofrequency waves. Therefore, the cooled-tip electrode is able to ablate a larger zone of tissue. A variation on this approach is the "wet" electrode (Berchtold; Tuttlingen, Germany). These are hollow electrodes that use an isotonic or hypertonic saline solution to perfuse the tissue being ablated. The saline is thought to enhance the zone of ablation by cooling the tip by increasing thermal conduction into the tissue because the liquid conducts heat better than the gas bubbles that otherwise form around the tip of the electrode, and by improving electrical conductivity because of an increased ion concentration (Ni *et al*., 2005). Other manufacturers have developed needle electrodes with expandable metal tines that deploy radially from the tip (Boston Scientific; Natick, Massachusetts, USA, and Angiodynamics [formerly RITA Medical Systems; Fremont, California, USA]). This formation dramatically increases the active surface of the electrode and the amount of tissue coagulated in a roughly spherical area around the electrode. A variation on this design is an electrode that deploys a coiled spring from the tip to increase the electric field produced (Invatec). Electrodes have also been designed to combine the advantages of saline perfusion with the advantages of expandable tines (Angiodynamics; formerly RITA Medical Systems). Larger RFA catheters capable of ablating larger tissue volumes are also being evaluated. At this time, there is little available data that suggests the superiority of any particular commercially available RFA device (Ni *et al*., 2005).

Even with technical advances allowing larger zones of tissue ablation, larger tumors require multiple deployments and overlapping zones of ablation to be adequately treated. In order to obtain a 1 cm margin of normal liver parenchyma, the maximum lesion diameter that can be treated with a single deployment of a 5 cm electrode is 3 cm. Mathematical models have demonstrated that to completely ablate a 4 cm tumor along with a 1 cm margin using a 5 cm RFA device, a total of six overlapping deployments of the electrode are necessary (Khajanchee *et al*., 2004). Larger tumors require even more overlapping ablations.

PATIENT SELECTION

At present RFA is generally used to treat colorectal liver metastases in patients who do not meet the criteria for surgical resectability, yet they do have disease confined to liver or have stable extra-hepatic disease. Radiofrequency ablation has also been used to expand the population of patients who may be treated with aggressive liver-directed therapy to attempt to increase survival and/or quality of life (Curley, 2001). For instance, some patients who were not candidates for surgical resection because of multi-lobar disease have been treated with a combination of RFA and hepatic resection (Pawlik *et al*., 2003) (Figure 27.1). Patients with liver tumors that

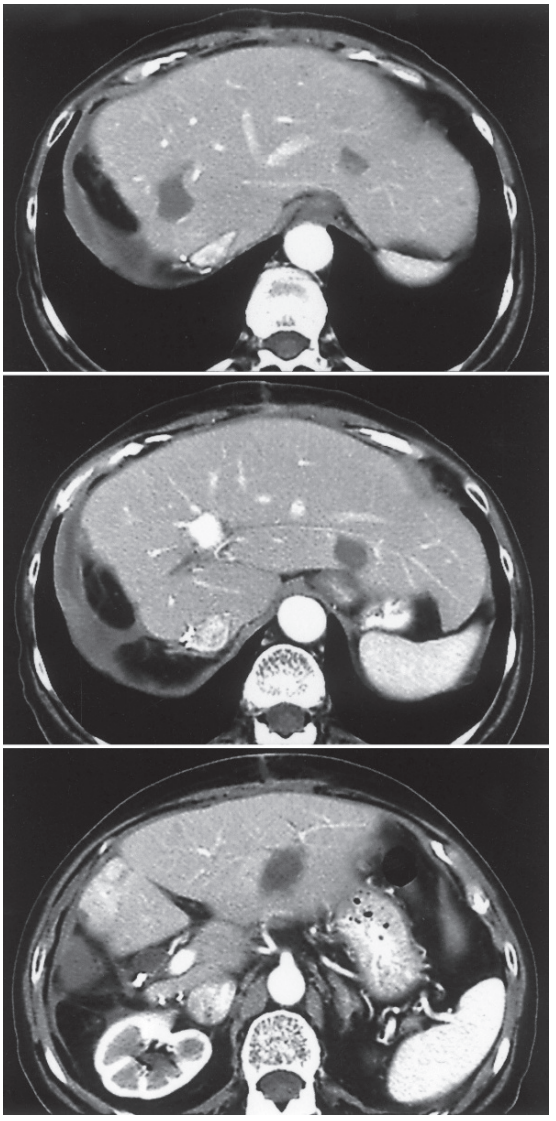

FIGURE 27.1. Postoperative CT scan images of a patient with bilateral, multi-lobar colorectal liver metastases. The patient underwent a right hepatectomy plus RFA of four left-sided liver metastases in an attempt to eradicate all metastatic disease, which was not possible with resection alone

are unresectable because of proximity to vital structures may still be candidates for RFA. The flowing blood in major hepatic vessels acts as a "heat sink" and protects the vessel wall from thermal injury, while allowing ablation of the neighboring tumor. However, the disadvantage of this heat dissipation is an increased risk of leaving viable tumor cells intact. For this reason, RFA is less effective for lesions adjacent to large vessels (Lencioni *et al*., 2000). To overcome this problem, some authors have described temporary occlusion of the tumors' blood supply (Rossi *et al*., 2000) or adjacent portal or hepatic veins (de Baere *et al*., 2002) during RFA. This approach should be used cautiously, as the incidence of portal vein thrombosis increases from 0.2% to 4.2% when a Pringle maneuver is used throughout the procedure (Mulier *et al*., 2002). Treatment of lesions in the area of the hepatic hilum risks thermal injury to the biliary tract and can lead to biliary strictures or fistulae. Radiofrequency ablation generally should be avoided to treat lesions in this area (Curley, 2001), although Elias *et al*. (2004) have described infusing the biliary tree with cooled saline during RFA for lesions closer than 6 mm to major bile ducts in an attempt to prevent stricture formation.

Patient selection criteria are still being refined. Larger lesions are less likely to be successfully ablated completely (Kuvshinoff and Ota, 2002; Mulier *et al*., 2005). Although some authors have found a significantly higher local recurrence rate when lesions were > 4 cm in diameter (Kuvshinoff and Ota, 2002), there is no strictly defined cut-off for size above which RFA would not be offered. Similarly, there is no absolute cut-off in terms of the number of metastases, although some authors would limit offering RFA to patients with greater than five or six lesions as the benefits are likely to diminish (Curley, 2001).

OPERATIVE TECHNIQUE

There are three commonly used approaches to perform RFA of colorectal liver metastases: percutaneous, open, and laparoscopic. There are advantages to each. The percutaneous approach is the least invasive method and can be performed as an outpatient procedure. It can be performed under general anaesthesia or with local anaesthetic with intravenous sedation. Subcapsular lesions where Glisson's capsule would be ablated or larger lesions requiring multiple passes of the probe may be better treated with general anaesthesia due to increased pain during the procedure. This minimal hospital stay and quick recovery make it an attractive option from an economic point of view, because the cost of such an approach is substantially less than the cost of open RFA or hepatic resection. On the other hand, percutaneous RFA does not appear to be as effective as open or laparoscopic approaches (Mulier *et al*., 2005). Percutaneous ultrasound may not detect as many lesions as intraoperative ultrasound (IOUS). In addition, ablation of subcapsular tumors can result in thermal injury to adjacent structures. Adjacent organs, such as diaphragm, stomach, colon, or small bowel, can be protected with laparoscopic or open approaches. In general these adjacent organs cannot be adequately protected with percutaneous approaches, although novel approaches such as injecting saline or carbon dioxide into the peritoneal cavity to displace and protect these organs have been described

(Kim *et al*., 2006; Raman *et al*., 2004). In some instances, lesions high in the dome of the liver may not be accessible via percutaneous approaches or may not be visualized by percutaneous ultrasound. In general, percutaneous RFA is indicated for patients being treated with palliative intent (Machi *et al*., 2001). It is also advantageous for those who are considered poor operative candidates or who refuse an open approach.

There are advantages to performing RFA as an open procedure with a laparotomy. Radiofrequency ablation used in conjunction with hepatic resection can expand the capabilities of surgery with curative intent to include patients who would not otherwise be candidates (Pawlik *et al*., 2003). Another advantage is that the sensitivity of IOUS is greater than that of other preoperative imaging modalities to detect hepatic lesions. IOUS has been shown to alter decision-making in the operating room in up to 73% of cases (Solomon *et al*., 1994). Elias *et al*. (2005) found unsuspected metastases with IOUS in 41% of patients who underwent hepatectomy for CRC liver metastases that would not have been detected and treated with percutaneous techniques. As technology improves and preoperative imaging becomes more sensitive, the value of IOUS may eventually decrease. However, it is currently considered the "gold standard" and patients treated percutaneously may receive less than optimal treatment if some metastases are missed. Other advantages of an open surgical approach include the ability to provide hepatic inflow occlusion to prevent dissipation of heat from the "heat-sink" effect of blood vessels, thereby increasing the likelihood of complete tumor necrosis (Curley, 2001). However, hepatic and portal venous occlusion has been performed during percutaneous RFA using percutaneous balloon catheter techniques (de Baere *et al*., 2002). As mentioned above, an open technique can permit protection of adjacent organs from unintentional thermal injury.

Laparoscopic RFA is another option that combines some of the advantages of the open approach with the benefits of a minimally invasive approach. The sensitivity of laparoscopic ultrasound is very close to that of traditional open IOUS (Tandan *et al*., 1997). Deep-seated lesions, particularly in segments 6, 7, or 8, can be difficult to reach (Machi *et al*., 2001). While a surgical approach (laparoscopic or open) has been shown to provide superior local recurrence rates than a percutaneous approach (Mulier *et al*., 2005), some authors have raised concerns that the recurrence rates achieved with laparoscopic RFA may be slightly inferior to those associated with an open approach (Kuvshinoff and Ota, 2002).

Radiofrequency ablation of colorectal liver metastases is most often performed under real-time ultrasound guidance. Ultrasound is used to guide placement of the RFA probe, which may have to be repeated many times for larger lesions and to monitor the zone of ablation. This technology has serious limitations. Radiofrequency ablation produces microbubbles as tissue is heated, which are hyperechoic on ultrasound imaging. This can obscure deeper portions of the lesions and make proper positioning difficult (Machi *et al*., 2001). For this reason, it may be prudent to ablate the deeper areas of the tumor first so that these areas are not obscured by prior more superficial ablations. In addition, ultrasound cannot differentiate necrotic from viable tumor (Solbiati *et al*., 2004). These weaknesses

can impair one's ability to achieve complete ablation of the lesion and may be a contributing factor to the high local recurrence rates seen in some series.

Newer imaging techniques are being investigated to overcome these limitations of ultrasound guidance. Contrast-enhanced ultrasound performed intraoperatively following ablation of metastases can detect residual tumor that is not seen on conventional ultrasound. This can then guide further deployments of the RFA probes in an effort to decrease recurrence rates. This technology has most often been studied in the setting of hypervascular hepatic malignancies such as hepatocellular carcinomas, but it has been shown to also be useful for colorectal metastases (Solbiati *et al*., 2004). Real-time computed tomography (CT) guidance is also being evaluated and may also allow improved detection of residual tumor (Vallone *et al*., 2003). Magnetic resonance (MR) imaging is a promising modality as well. It has the ability to monitor the thermal effects of RFA in real-time by following the effects of temperature changes in the tissue. This allows immediate detection of inadequately treated tumor foci and subsequent interactive repositioning of the radiofrequency electrode during therapy (Clasen *et al*., 2006). Contrast enhancement can provide further information regarding the completeness of ablation. However, using CT or MR guidance for RFA is more time consuming. While real-time CT and MR guidance have most often been used with a percutaneous approach, the feasibility of performing complex open abdominal surgery along with RFA in a specially designed intraoperative MR suite was described (Bathe *et al*., 2006).

FOLLOW-UP AFTER SURGERY

Standard follow-up protocols include serial CT or MR imaging in addition to serum carcinoembryonic antigen levels to assess for recurrent disease. These imaging modalities demonstrate a nonenhancing zone of ablation surrounded by an enhancing rim (Figure 27.2). This enhancing rim is thought to be a physiologic response to thermal injury and usually disappears after several months. This benign periablational enhancement should be differentiated from residual tumor. The former is thin (generally 1–2 mm), concentric, and regularly shaped with smooth inner margins. Residual tumor often grows in nodular or eccentric patterns (Lencioni *et al*., 2004) (Figure 27.3). On MR imaging, residual tumor is indicated by nodular or irregular enhancement with hypointensity on unenhanced T1 images and hyperintensity on T2 images (Clasen *et al*., 2006). Although less widely available, 18-fluorodeoxyglucose positron emission tomography (FDG-PET) may be a more accurate method of following patients (Ravikumar *et al*., 2000). In a subgroup of patients in a series reported by Joosten

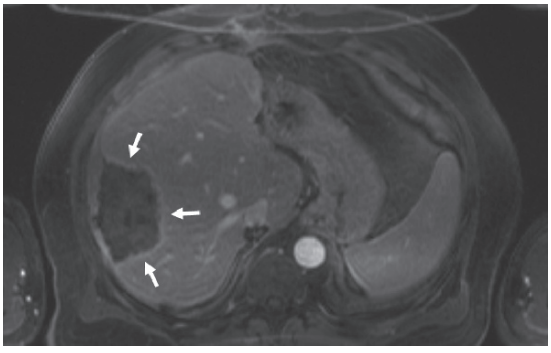

FIGURE 27.2. MR image showing the post-ablation appearance of a colorectal liver metastasis. There is a thin, evenly distributed rim of enhancement surrounding the ablation zone (white arrows)

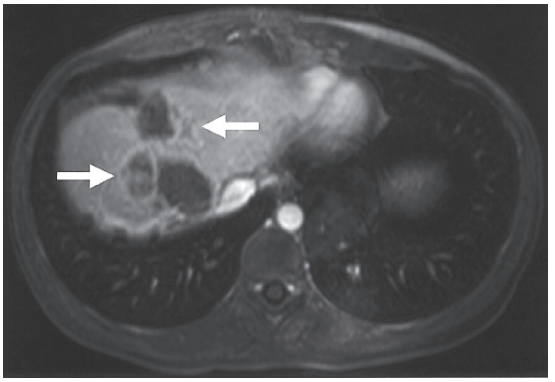

FIGURE 27.3. Follow-up MR image of two previously ablated colorectal metastases in the dome of the liver. The arrows demonstrate adjacent areas of irregular, nodular enhancement that is indicative of local tumor recurrence

et al. (2005), FDG-PET performed at 3 weeks predicted six of seven local recurrences, whereas CT predicted none of them.

SAFETY

Radiofrequency ablation appears to be a relatively safe procedure, with low rates of complications and mortality. Livraghi *et al*. (2003) reported a mortality rate of 0.3% and a major complication rate of 2.2% in a series of 2,320 patients undergoing RFA. The most common complications were intraperitoneal hemorrhage (0.5%), tumor seeding along the needle tract (0.5%), liver abscess (0.25%), gastrointestinal perforation (0.22%), and hemothorax (0.13%). Studies of another series of 312 patients by de Baere *et al*. (2003) report a mortality rate of 1.4% with a major complication rate of 5.7%. Hepatic abscess following RFA, while a very rare complication, was seen much more commonly in patients who had prior bilioenteric anastomosis. Shibata *et al*. (2003) reported an odds ratio of 36.4 for abscess formation when a prior bilioenteric anastomosis was performed. Elias *et al*. (2006) reported that the risk of developing a hepatic abscess when prior biliary stenting or bilioenteric anastomosis was performed was 40–50%. Post-radiofrequency ablation syndrome is seen quite commonly. It can be seen in approximately one third of patients after RFA (Wah *et al*., 2005). This is a self-limiting syndrome consisting of flu-like symptoms and fever. Treatment is supportive. Symptoms peak around day 3 and generally resolve by day 10. Longer-lasting fevers should prompt a search for other causes.

OUTCOMES

There have been several recent studies evaluating the effectiveness of RFA as a treatment modality for both primary and secondary malignancies of the liver. The earliest studies examined the ability of RFA to achieve complete necrosis of the tumor, while more recent studies have begun to report longer-term outcomes. Many studies report heterogeneous populations where patients with primary hepatic malignancies are combined with patients with various types of metastases. This makes it difficult to draw firm conclusions about the effectiveness of RFA in patients specifically with colorectal metastases. The existing literature concerning RFA for colorectal liver metastases is limited. Most of the existing publications consist of uncontrolled case series. Thus, firm conclusions cannot be drawn and direct comparisons to existing treatments are quite difficult to make. Nonetheless, the current evidence is summarized below.

RATES OF COMPLETE TUMOR NECROSIS

Many studies have assessed the completeness of ablation with post-procedure imaging with either CT scans or MR imaging. However, these follow-up images often reveal an area of necrotic liver tissue and it may be impossible to tell if viable tumor cells remain or whether the lesion is truly ablated completely. The inability to accurately predict complete tumor necrosis on a single follow-up study was demonstrated by coexisting high rates of complete tumor ablation and high rates of local recurrence in some series (Solbiati *et al*., 2001; White *et al*., 2004). Rates of complete tumor ablation for colorectal metastases have ranged from as high as 98% (Solbiati *et al*., 2001) to as low as 50% (de Baere *et al*., 2000). At least part of this variability may be related to the subjective nature of this outcome and the heterogeneity between these series. Although, it has not been widely used, 18-fluorodeoxyglucose positron emission tomography may be more predictive than CT or MRI for detecting the presence of viable tumor cells in the ablated lesion (Joosten *et al*., 2005). The main benefit of checking whether complete ablation has been achieved may be to determine the need for an early repeat ablation. Contrast-enhanced ultrasound is another technique that can be used to assess completeness of ablation and guide further deployments of the RFA probe (Solbiati *et al*., 2004). This can be performed at the end of the procedure.

RECURRENCE RATES

Perhaps a more important outcome is the rate of tumor recurrence documented by tumor growth at the previous ablation site as seen on serial follow-up imaging (rather than a single post-procedure study). One of the biggest concerns regarding the effectiveness of RFA that has been raised is the high recurrence rates seen in some series. Local recurrence in this setting refers to tumor growth arising from the same site as a previously ablated lesion, indicating that viable tumor cells have been left behind.

While some authors have reported that local recurrence rates with RFA are not significantly different than those with anatomic or wedge resections of the liver (Elias *et al*., 2004), the current literature reports a wide range of local recurrence rates for colorectal liver metastases treated with RFA. These rates range from 5% (Iannitti *et al*., 2002) to as high as 39% (Solbiati *et al*., 2001; White *et al*., 2004). However, several series report local recurrences <10% (Abdalla *et al*., 2004; Iannitti *et al*., 2002). The lower recurrence rates may be a function of patient selection (i.e., small lesions) or short follow-up in some instances.

These higher local recurrence rates are cause for concern, indicating that RFA may not be as effective in completely destroying all viable tumor cells as some authors had hoped. Local recurrence has been clearly linked to tumor size (Kuvshinoff and Ota, 2002) and some investigators suggest that RFA should not be done for lesions larger than 4 or 5 cm (Gillams and Lees, 2005; van Duijnhoven *et al*., 2006). Improved local control has also been reported when using an open approach compared to a percutaneous one (Kuvshinoff and Ota, 2002; Mulier *et al*., 2005). In addition, central lesions may be more difficult to completely ablate (van Duijnhoven *et al*., 2006).

One of the weaknesses of RFA that may lead to local recurrence is that it relies on real-time imaging to guide proper probe placement. Currently, most authors use real-time ultrasound to guide RFA probe placement. The hyperechoic microbubbles that occur as tissue is heated obscure deeper portions of the lesions and make proper positioning difficult (Machi *et al*., 2001). Although not widely available, intra-operative MRI guidance (Clasen *et al*., 2006) or contrast-enhanced ultrasound (Solbiati *et al*., 2004) may be more accurate imaging methods to detect areas of tumor which may have been incompletely ablated and allow immediate re-treatment. Advances are being made in the design of the probes. Ahmad *et al*. (2006) found significantly improved rates of local recurrence and disease-free survival in patients with colorectal liver metastases when treated with newer-generation RFA probes.

Local recurrences should be distinguished from the development of new liver lesions. The development of new liver lesions may be as high as 57% in some patients (Solbiati *et al*., 2001), and does not necessarily represent a failure of RFA. It is likely a reflection of the underlying disease process and patient selection. Patients who undergo RFA generally have more advanced disease than patients who undergo hepatic resection and may be at higher risk of developing new metastases elsewhere in the liver.

Part of the reason that RFA may not be able to match the recurrence rates of hepatic resection is that hepatic resection decreases the amount of liver parenchyma at risk for new metastases. Abdalla *et al*. (2004) raised some interesting concerns regarding the development of new liver lesions. They compared the outcomes of 190 patients who underwent hepatic resection with curative intent to 158 patients where complete resection was not possible (101 had RFA plus resection and 57 had RFA alone). Thirty-five percent of those who had RFA alone developed new liver lesions compared to 9% of those who had hepatic resection with curative intent, suggesting that although RFA was less successful in local control (9% vs. 2% for resection), a major benefit of hepatic resection may be the development of fewer new lesions in the liver. This may be partially because resection removes liver parenchyma at risk for future metastases. Of the 190 patients who underwent resection, 122 had at least four Couinaud segments resected (31 had extended hepatectomies) and 22 patients had additional contralateral resections, so the development of new metastases may be roughly proportional to the amount of residual liver parenchyma. This should be interpreted cautiously, however, since factors such as patient selection could also explain the difference in recurrence rates.

SURVIVAL

The ultimate outcome measure in considering whether RFA may become an alternative to resection in those amenable to resection in the future will be long-term survival. To date, long-term survival remains limited. Many series have combined patients with primary and secondary hepatic malignancies. The available series reporting long-term survival of patients with colorectal liver metastases undergoing RFA is summarized in Table 27.1. In all of these studies, patients were considered to have unresectable disease on the basis of general health considerations, refusal of surgery, or technical reasons such as

TABLE 27.1. Survival rates associated with radiofrequency ablation of colorectal liver metastases.

Author	Year	No. Patients	Approach	Mean No. lesions	Mean lesion diameter (cm)	Median follow-up (mos.)	Local recurrence (%)	1-year survival (%)	2-year survival (%)	3-year survival (%)	5-year survival (%)
Solbiati *et al.*	2001	117	P	1.5	3.2	6–52	39	93	69	46	
Iannitti *et al.*	2002	52	O, L, P	2.7	5.2	20		87	77	50	
Oshowo *et al.*	2003	25	P	1a	3.0					53	
White *et al.*	2004	30	P	1.9	3.0	17		75	45		
Abdalla *et al.*	2004	57	O	1[a]	2.5[a]	21	9			37	
Lencioni *et al.*	2004	423	P	1.4	2.7	19	25	86	63	47	24
Gillams and Lees	2005	167	P	4.1	3.9			99[†]		58[a]	30[b]
Joosten *et al.*	2005	28	O	3[c]	2[c]	25	6	93	75	46	
Machi *et al.*	2006	100	O, L, P	3.5	3.0	25	7	90		42	31
Aloia *et al.*[d]	2006	30	O, P	1a	3.0	31	37			57	27
Abitabile *et al.*	2007	47	O, P	3.1	2.4	21	9	88	80	57	21

[a]Reported figures are median values from subset of 57 patients who underwent RFA alone.
[b]These survival figures were taken from subset of 73 patients with less than five lesions that were all less than 5 cm in diameter.
[c]Reported figures are median values.
[d]The patients in this series were likely also included in the series from Abdalla *et al.* (2004) above.

poor liver reserve, bilateral distribution of tumor, or proximity to major vascular structures.

Several of these studies have reported favorable survival rates and this has prompted some authors to call for randomized controlled trials comparing RFA to hepatic resection (Gillams and Lees, 2005; Oshowo *et al*., 2003). Oshowo *et al*. (2003) called for randomized trials after reporting a series of 45 consecutive patients presenting to their center with solitary colorectal liver metastases. Twenty of these patients were candidates for liver resection and underwent the procedure. They served as a control group to compare with patients who underwent RFA. The other 25 were not candidates for resection because of proximity to major vascular structures (9 patients), medical comorbidity (9 patients), and extra-hepatic disease (7 patients). These 25 patients underwent percutaneous RFA. They had mean age of 57 years and the median size of lesion was 3 cm (range 1–10 cm). There were no mortalities, but one patient developed a pleural effusion requiring treatment. The 3-year survival for this group was 52.6%. The 20 patients who underwent hepatic resection had a mean age of 63 and most (16 patients) had metachronous lesions. The 3-year survival for this group was 55.4%, almost identical to the patients who underwent RFA. This was impressive, because the patients treated with RFA were not considered candidates for resection and likely had a higher burden of disease. However, details regarding adjuvant chemotherapy were not described.

Similarly, Gillams and Lees (2005) called for randomized trials after reporting the outcomes of 167 patients with inoperable colorectal liver metastases treated with RFA. Patients had an average of 4.1 lesions each, with an average diameter of 3.9 cm. Fifty-one patients had either treated or stable extra-hepatic disease or had perforated primary tumors that placed them at high risk of carcinomatosis. The majority of patients received chemotherapy (80%), and 16% had RFA in conjunction with hepatic resection. The median survival of the entire group was 32 months with 1-, 3-, and 5-year survival rates of 91%, 40%, and 17%, respectively. The major and minor complication rates were 4% and 6%, respectively. In a subgroup analysis, 73 patients who had less than five lesions and no lesion larger than 5 cm had better survival, with a median of 38 months and 1-, 3-, and 5-year survival rates of 99%, 58%, and 30%, respectively. This suggests that stricter patient selection criteria may result in improved survival.

While these studies have shown promising results and some authors have suggested that these results warrant randomized trials, the findings of Abdalla *et al*. (2004) were much less optimistic. These authors have cast serious doubt on whether RFA will prove to be an alternative to hepatic resection. As mentioned above, they compared outcomes of 190 patients who underwent hepatic resection with curative intent to 158 patients in whom complete resection was not possible (101 patients underwent resection plus RFA and 57 who underwent RFA alone) and to 70 patients without extra-hepatic disease who underwent only biopsy or hepatic arterial infusion pump placement at the time of exploratory laparotomy ("chemotherapy only"). Among the 57 patients who underwent RFA alone, the median number of lesions was 1, and the median tumor size was 2.5 cm. Eleven of the 190 patients who underwent hepatic

resection and 27 of the 158 patients who underwent RFA with or without resection also had chemotherapy via hepatic artery infusion pump. Details regarding systemic chemotherapy were not included. Those who underwent resection with curative intent had a 5-year survival of 58%. Although the high survival rate at 5 years may indicate a highly selected group of patients, the 3-year survival rate of 73% was significantly better than that for patients who underwent RFA alone or in combination with resection (3-year survival of 37% and 43%, respectively). Patients who received RFA did have significantly higher survival than patients who had chemotherapy only. The rates of distant metastases were similar between all surgically treated groups, but local recurrences and new lesions within the liver were more common with RFA. Since the development of distant metastases was the same between groups, the survival difference may have been partly due to the difference in local recurrence and development of new liver lesions.

An updated report from the same center (Aloia *et al*., 2006) raised further concerns regarding the effectiveness of RFA. These authors prospectively compared the outcomes of 150 patients with solitary colorectal liver metastases treated by hepatic resection to the 30 patients with solitary colorectal liver metastases treated by RFA in the above study by Abdalla *et al*. (2004) after a longer follow-up. After a median of 31 months, 5% of patients who underwent liver resection developed local recurrence. By this time, the proportion of patients treated with RFA that had recurrence at the ablation site had risen from 9% to 37%. Furthermore, the 5-year overall survival was significantly worse for patients who had RFA compared to those who had resection, even though patient characteristics and the proportion of patients receiving adjuvant chemotherapy were similar between groups. This series suggests that in patients with comparable burden of disease (solitary metastases), the outcomes following RFA do not appear to match those following hepatic resection. In fact, the authors from this center suggested that a randomized controlled trial comparing the two treatment modalities would be unethical (Abdalla and Vauthey, 2006).

In summary, the effectiveness of RFA remains controversial, and in the absence of randomized studies several questions remain. Patient selection is an obvious concern when interpreting these data. While all patients have been considered to have unresectable disease, it should be kept in mind that "unresectable" can be a relative term. Different surgeons may have different opinions as to what constitutes unresectable disease. Publication bias may be present and patients with relatively good prognoses may have been chosen to be included in these studies. Another confounding variable is that many of these patients received systemic chemotherapy, which may have been responsible for some of the favorable survival data. The series reporting outcomes following RFA of colorectal liver metastases are quite heterogeneous. Patient numbers in these series range from 25 to 423 and include patients who had open, laparoscopic, and percutaneous approaches. Also, lesion size, number, and distribution vary widely between series, which creates further problems when pooling results or drawing conclusions across studies.

CURRENT STATUS OF RADIOFREQUENCY ABLATION FOR COLORECTAL LIVER METASTASES

Presently, there is still a scarcity of evidence concerning the long-term outcomes after RFA of colorectal metastases. There were no well-controlled or randomized studies, and these series must be considered level III evidence. The available studies are heterogeneous in terms of patient selection criteria, number of lesions, and size of lesions, so drawing conclusions across these studies and pooling data are difficult. There remain many unresolved issues regarding patient selection criteria in terms of size and number of lesions, the optimal method of delivery (open, laparoscopic, or percutaneous), and optimal methods of image guidance.

Reported 5-year survival rates range between 21% (Abitabile *et al*., 2007) and 31% (Machi *et al*., 2006), which is impressive considering these patients were not considered to have resectable disease. A major cause for concern is the fairly high rate of recurrent disease reported in some studies, both locally and at other sites in the liver. This reinforces the generally held view that hepatic resection remains the standard of care when feasible, and that the results of longer-term survival data are needed. Radiofrequency ablation for colorectal liver metastases does appear to offer improvements in survival beyond what can be achieved with systemic chemotherapy alone (Abdalla *et al*., 2004). It does appear to have a valuable role in treating patients with unresectable disease, and may also be used in conjunction with hepatic resection to extend the limits of resection. Currently, RFA cannot be considered equivalent to hepatic resection.

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28
Anal Squamous Cell Carcinomas: Diagnosis Using p63 Immunohistochemistry

Scott R. Owens

INTRODUCTION

Anal Carcinoma

Anal canal carcinomas are relatively uncommon, accounting for only about 1.5% of gastrointestinal cancers in the United States according to Ryan *et al*. (2000). Fenger *et al*. (2000) indicate that approximately 80% of these are squamous cell carcinomas, which include keratinizing, non-keratinizing and basaloid (cloacogenic) subtypes. Iacobuzio-Donahue (2004) pointed out an important distinction regarding anal carcinomas: namely, that carcinomas arising above the dentate line must be distinguished from those arising below it. The former are much more common, arise from the anal transitional zone, and may be of several types, including adenocarcinoma, neuroendocrine carcinoma and basaloid (cloacogenic) squamous carcinoma. By contrast, those arising below the dentate line are nearly always squamous cell carcinomas.

Anal squamous carcinomas are very frequently related to chronic infection with the human papilloma virus (HPV), and usually occur in the sixth or seventh decade of life. They are about twice as common in females as in males. The age of incidence can be significantly younger in patients who are immunocompromised, and the advent of diseases such as HIV/AIDS as well as the increasing use of immunosuppressive therapy for patients with solid organ transplants, inflammatory bowel disease and collagen vascular diseases has provided the background for an increasing incidence of HPV infection and anal squamous cell carcinomas over the past several decades.

The diagnosis of carcinomas in the anus can be made difficult by both anatomical and histological considerations. The spatial characteristics of such a restricted area result in the obliteration of anatomical landmarks by even relatively small tumors. Furthermore, the proximity of different tissue types above and below the dentate line means that different subtypes of carcinoma must be considered, and carcinomas originating in the lower rectum can extend into the anal canal as well. Further difficulty can arise when the diagnostic material consists of a small biopsy obtained endoscopically or transanally. The clinical physician may be confused by altered anatomy, and the resultant biopsies

are small and often suffer from crush artifact. Such biopsies are often the first diagnostic specimen received in the pathology laboratory.

As suggested above, the differential diagnosis of carcinomas in the anal canal most commonly includes squamous cell carcinoma, poorly-differentiated adenocarcinoma, and well-differentiated neuroendocrine carcinoma/carcinoid tumor. The latter entities may arise within the anal canal above the dentate line or, commonly, may extend into the anus from primary sites in the rectum. Less commonly, such tumors may be metastatic from other sites. In larger excision or resection specimens, the diagnosis can be aided by observing morphological clues. For example, squamous eddy or so-called "keratin pearl" formation implies squamous differentiation, lumen or tubule formation is characteristic of adenocarcinoma, and a distinctive stippled or "salt-and-pepper" nuclear chromatin pattern can suggest neuroendocrine differentiation. Many squamous carcinomas are accompanied (or preceded) by intraepithelial dysplasia and/or features of HPV infection, so a search for surface epithelium with cytologic atypia or koilocytic changes may bear fruit.

Unfortunately, however, all of these carcinomas can look remarkably similar in a small biopsy, often appearing as small nests or collections of nondescript-appearing tumor cells. Basaloid squamous cell carcinomas can have dark, homogeneous nuclear chromatin resembling that of neuroendocrine carcinomas. Nests of adenocarcinoma cells without lumen formation or mucin production can be essentially indistinguishable from other types of carcinoma. Therefore, a panel of immunohistochemical stains is very often utilized as a diagnostic adjunct. For the diagnosis of squamous cell carcinoma, these panels most often rely on negative staining with markers of colorectal adenocarcinoma (such as CDX-2 and cytokeratin 20) and neuroendocrine differentiation (such as chromogranin and/or synaptophysin), accompanied by positive staining with one or more cytokeratins (typically cytokeratins 5/6) suggesting squamous origin or differentiation. As noted by Williams *et al*. (1997), however, this approach is often complicated by the loss of keratin expression in poorly-differentiated squamous carcinomas. Cury *et al*. (2000) point out that cytokeratins 5/6 can be positive in other types of carcinoma, including up to 30% of colorectal adenocarcinomas. Owens and Greenson (2007) found that 26% of colorectal adenocarcinomas and 19% of colorectal neuroendocrine neoplasms stained with these cytokeratins.

Accurate diagnosis of carcinomas in the anal region is imperative, because of therapeutic implications. According to Ryan *et al*. (2000), most carcinomas originating in the anus are treated by chemoradiation regimens, while adenocarcinomas and neuroendocrine carcinomas from the rectum are treated surgically. Therefore, an accurate diagnosis and assessment of site of origin are imperative for the informed development of a treatment plan.

p63

The p63 gene resides on the long arm of chromosome 3 at 3q27-28, according to Kaelin (1999), and Little and

Jochemsen (2002). This gene is part of the p53 gene family, and participates in epithelial proliferation and differentiation. It is expressed in six or more isoforms, with some containing a transcription activating (TA) domain, and others lacking this domain (termed ΔN isoforms). The TA isoforms can activate target gene transcription, inducing arrest of the cell cycle and apoptosis. In contrast, the ΔN isoforms cannot activate transcription, instead acting in a dominant negative fashion and inhibiting gene activation by both the TA forms of p63 and by p53. While p53 is an important tumor suppressor and p53 mutations lead to carcinogenesis in the Li-Fraumeni syndrome, p63 is thought to participate in development, and has been implicated in developmental abnormalities and syndromes.

As noted by Di Como *et al*. (2002), immunohistochemical antibody to p63 is often utilized in the practice of diagnostic pathology when evaluating prostatic adenocarcinoma. In this setting, normal prostate glands are lined by a p63-positive layer of basal cells, whereas prostatic adenocarcinoma lacks a basal cell layer. Hall *et al*. (2000) and Reis-Filho *et al*. (2003) indicate that the p63 protein has also been found in squamous cell carcinomas in a number of anatomical sites, including the head and neck region, the lung, the uterine cervix, and the esophagus. It is also expressed in certain breast carcinomas (particularly metaplastic carcinoma) according to Koker and Kleer (2004), and in urothelial carcinomas. Wang *et al*. (2002) note that squamous carcinomas are frequently found to have genomic amplification on the long arm of chromosome 3, where the p63 gene is found.

p63 IMMUNOSTAINING IN ANAL CARCINOMA

Utility

Immunohistochemical staining for p63 is of use in the diagnosis of anal carcinomas, as reported by Owens and Greenson (2007). This study utilized p63 in the differentiation of squamous cell carcinoma from the two other neoplasms commonly encountered in the differential diagnosis of anal tumors (adenocarcinoma and neuroendocrine carcinoma/carcinoid). The outcome indicated 98% specificity and 92% positive predictive value for the p63 immunohistochemical stain in squamous carcinomas (Figure 28.1). This compared to a specificity and positive predictive value of 78% and 69%, respectively, for cytokeratins 5/6. Basaloid squamous cancers (also known as "cloacogenic" carcinomas), were also positive for p63, potentially simplifying their discrimination from neuroendocrine carcinomas, which can have a similar histological appearance. The outcome was the same for both resection and biopsy specimens, and no additional utility was gained by combining p63 with cytokeratins 5/6. Chetty *et al*. (2005) reported two cases of basaloid squamous cell carcinoma of the anal canal that had an unusual appearance mimicking adenoid cystic carcinoma. Both of these cases had diffuse staining with p63.

Advantages

The use of p63 immunostaining provides several advantages over other stains commonly used in the diagnosis of anal carcinomas. As mentioned above, cytokeratins 5/6, which are often utilized as mark-

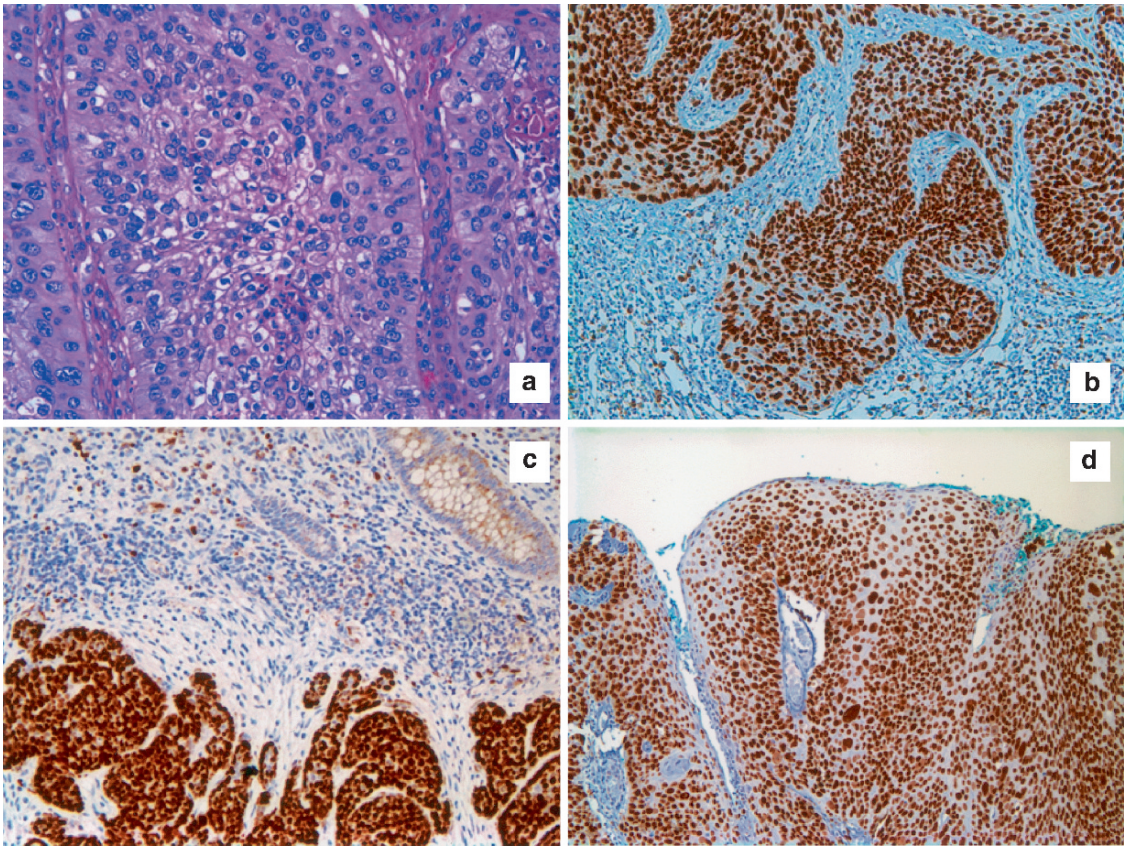

FIGURE 28.1. (a) Poorly-differentiated squamous cell carcinoma (400x). Note lack of morphological evidence for squamous differentiation and similarity to other poorly-differentiated carcinomas. (b–d) Immunohistochemical staining with antibody to p63 in invasive squamous cell carcinoma, basaloid squamous cell ("cloacogenic") carcinoma, and high-grade anal intraepithelial neoplasia (AIN III), respectively (100x). Note strong nuclear staining

ers of squamous differentiation, can also stain adenocarcinoma and occasionally neuroendocrine neoplasms, both entities in the differential diagnosis of poorly-differentiated anal tumors. Furthermore, these keratins also mark dermal adnexal structures, and often stain the cytoplasm so strongly as to totally obscure any cytological detail. This can potentially complicate the distinction between an adnexal structure in the perianal skin and a nest of invasive carcinoma, especially in a small biopsy from the anal region.

In addition to positive staining in squamous carcinomas, p63 is also potentially useful in the diagnosis of squamous dysplasia. As noted earlier, the vast majority of anal squamous carcinomas are HPV-related. Therefore, HPV-induced dysplasia is a regular finding in biopsies from the anal region that contain squamous carcinoma, and is a relatively common rea-

son for biopsy even when no carcinoma is present, such as in anal condylomata or in follow-up biopsies of previously-diagnosed anal intraepithelial neoplasia (AIN).

The aforementioned study also noted that an additional potential utility of p63 staining was its tendency to highlight dysplastic squamous epithelium as well as invasive squamous carcinoma. As such, non-dysplastic anal squamous mucosa exhibited staining of only the basal layer with p63, while dysplastic epithelium demonstrated p63 staining that increased progressively in thickness with the degree of dysplasia. Thus, for example, high-grade squamous intraepithelial lesion/AIN III was decorated with full-thickness p63 staining. This finding illustrates another advantage of p63 over cytokeratins 5/6, which stain both normal and dysplastic squamous epithelium equally and uniformly. Thus, a potential future use for the p63 immunostain is in the diagnosis and follow-up of HPV-induced squamous dysplasia, and it may also prove useful in tissues outside the anal canal, such as the uterine cervix and the vulva.

PROCEDURE AND INTERPRETATION

A variety of protocols for p63 immunohistochemical staining exists, most utilizing the 4A4 clone or a variant thereof. Owens and Greenson (2007) described using the DakoCytomation® automated staining system (DakoCytomation, Inc.; Carpenteria, CA), but protocols using the Ventana® system are also available, providing functionality in most laboratories capable of routine immunohistochemical staining.

According to data provided by the reagent manufacturer, the 4A4 clone of the p63 antibody is a mouse anti-human IgG2a isotype with a Kappa light chain, which reacts with the first 205 amino acids of the p63 protein. All isotypes of p63 are recognized by the antibody, which is produced using a recombinant protein derived from amino acids 1–205 of human ΔNp63. Once the primary antibody has reacted with the tissue to be assayed, binding is detected using goat anti-mouse immunoglobulin conjugated to a peroxidase-labeled polymer. The addition of diaminobenzidine, results in the generation of a chromogen in nuclei when p63 is expressed in the target tissue.

Prostate tissue is a suitable positive control for the p63 staining process. Normal prostate tissue exhibits p63 staining of the basal cell layer of the prostatic glands. The use of a section of tissue also containing prostatic adenocarcinoma (in which basal staining by p63 is lost) provides an additional assurance of the specificity of the antibody. Positive basal staining is also seen in a variety of squamous epithelium, such as in the uterine cervix and skin. Some tissues can exhibit non-specific cytoplasmic staining (Figure 28.2a). Therefore, unambiguous nuclear staining should be required for designation of tissue positivity.

UNIQUE CONSIDERATIONS

The use of p63 as a diagnostic adjunct in anal carcinomas requires some additional caution. Owens and Greenson (2007) reported that in addition to the staining shown by squamous cell carcinomas, two classic appendiceal carcinoid tumors

exhibited nuclear p63 staining as well. The restriction of this staining to neuroendocrine tumors from the appendix, a product of the embryonic mid-gut, could be related to variations in p63 expression throughout the gut. This raises the possibility that some neuroendocrine tumors in the anus may rarely stain with p63. Furthermore, the staining of the basal layer of both normal squamous epithelium and the anal transitional zone (Figure 28.2b), as well as more widespread staining of dysplastic squamous mucosa, mean that the use of p63 for subclassification of carcinomas should be carefully restricted to cases in which the diagnosis of invasive carcinoma has already been made. This avoids the pitfall of misdiagnosing squamous dysplasia as invasive squamous cell carcinoma in the setting of strong p63 staining, and should be distinguished from the potential use of p63 in the detection and grading of HPV-related dysplasia/AIN when invasive carcinoma is not suspected.

As mentioned previously, p63 is expressed in squamous carcinomas from other sites such as the head and neck, lung and uterine cervix, as well as in certain other carcinomas, such as urothelial and breast. This raises the possibility, albeit relatively

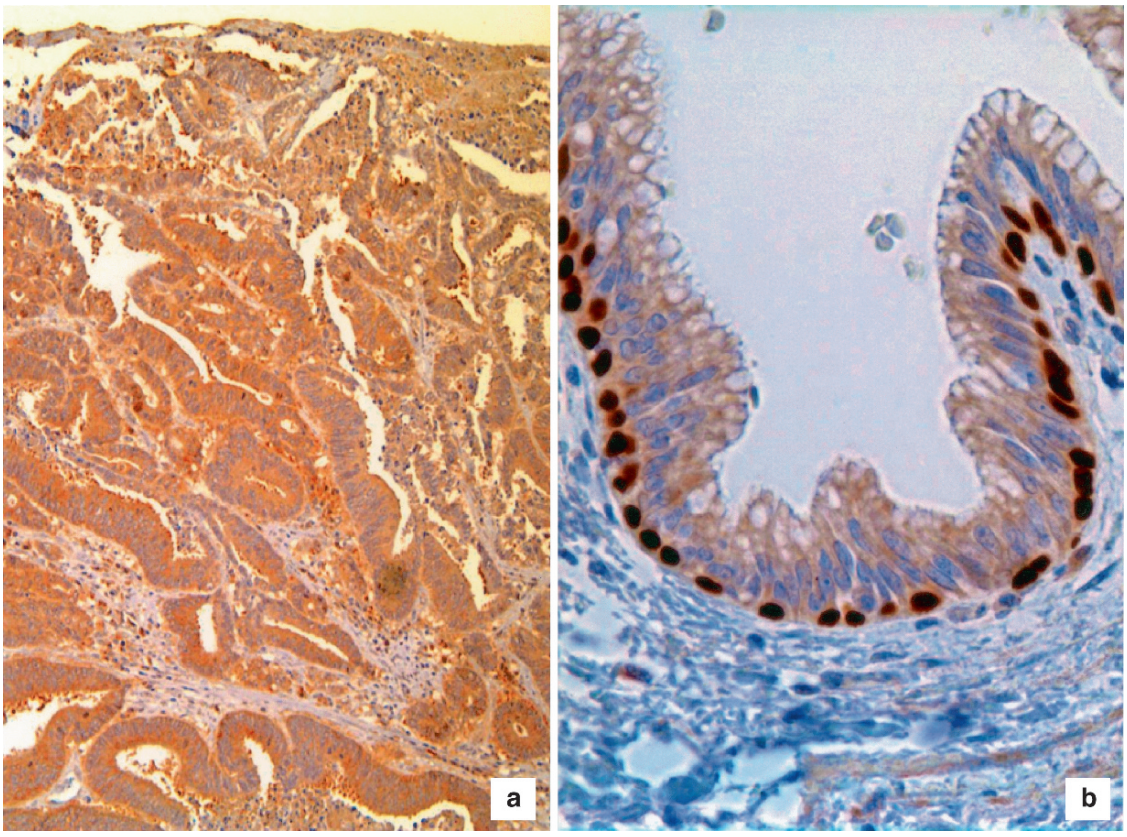

FIGURE 28.2. (A) Invasive adenocarcinoma with p63 immunohistochemical stain (100x). Note non-specific cytoplasmic staining, but lack of any nuclear reactivity. (B) Anal transitional zone, showing only basal reactivity with p63 immunohistochemical stain (400x)

unlikely, that carcinomas either metastatic to or directly extending into the anal region from other sites could provide a point of confusion when p63 is utilized as a diagnostic tool. While breast or lung carcinoma metastatic to the anal canal would be a rare occurrence, direct extension of a p63-positive urothelial or cervical carcinoma into the anus is quite plausible, and may result in confusion when p63 immunostaining is used in the diagnostic work-up.

SUMMARY

The diagnosis of carcinomas in the anal canal is fraught with potential difficulty. Most often, the first specimen received in the pathology laboratory in such cases is a small biopsy, susceptible to crush artifact and taken from a confined, anatomically confusing space. While most anal tumors are HPV-associated squamous cell carcinomas, the differential diagnosis usually includes neuroendocrine tumors, and poorly-differentiated adenocarcinoma and biopsies can contain nests of poorly-differentiated cells that can be difficult to sub-classify. A variety of immunohistochemical stains has been utilized in an attempt to correctly diagnose anal tumors, and recent studies have indicated that the p63 immunostaining offers a single modality that can identify squamous carcinomas with high specificity.

The p63 immunostain has advantages over other commonly-used stains, including being relatively simple to interpret, preferentially staining carcinomatous and dysplastic squamous epithelium without diffusely staining normal mucosa, and highlighting HPV-associated squamous dysplasia with increasing reactivity proportional to the degree of dysplastic change. As its use increases, caution must be exercised, since it can also stain squamous carcinomas from other sites (such as the head and neck or uterine cervix), as well as other types of carcinoma including metaplastic breast carcinoma and urothelial carcinoma. When the diagnosis of invasive carcinoma in anal biopsies has been established; however, the p63 immunostain provides a very helpful tool in its sub-classification.

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29
Molecular and Clinicopathologic Features Which Predict Outcome in Patients with Anorectal Melanoma

Hak-Mien Quah and Martin R. Weiser

INTRODUCTION

Anorectal melanoma is a rare and lethal tumor associated with extremely poor prognosis. Representing < 1% of all melanomas and 4% of anal canal malignancies, (Klas *et al*., 1999), anal melanoma can be a difficult clinical diagnosis because patients present with nonspecific signs and symptoms such as rectal bleeding or anal pain. Furthermore, lesions may be amelanotic, requiring biopsy for definitive diagnosis. Although the great majority of patients initially present with what appears to be curable localized disease, undetected metastases are common and mean survival is a mere 2 years. Even with aggressive surgical treatment, > 80% will die of distant metastatic disease within 5 years.

This chapter highlights the epidemiological, clinicopathologic, and molecular features of anorectal melanoma, and discusses the differences between this lethal disease and the much more common and curable cutaneous form. Observations regarding outcomes after surgical resection will also be reviewed, as will predictors of survival. Additionally, the recent discovery that the *KIT* oncogene may be aberrantly activated in a subset of patients, thus raising the possibility of developing molecular therapy for anorectal melanoma, will be addressed.

EPIDEMIOLOGY

The American Cancer Society estimates that ~59,940 new melanomas will be diagnosed in the United States in 2007; ~8,110 individuals will die of disease. The overall incidence continues to rise.

All melanomas originate from melanocyte, a pigmented, dendritic-like cell found in various anatomic sites including the base of the epidermis, the eye, oropharyngeal epithelium, nasal cavity, vagina, urinary tract, and anus. Cutaneous melanomas are far more common than noncutaneous (i.e., ocular and mucosal), accounting for > 90% of all melanomas. (While cutaneous melanomas account for only ~3% of all diagnosed skin cancer malignancies, they are nevertheless the cause of a vast majority of skin cancer-related deaths.) Ocular melanoma accounts for 5%, melanoma of unknown origin for 2%, and mucosal melanoma for 1%, respectively. Head and neck, anorectal, female genital, and urinary tract tumors account for 55%, 24%,

18%, and 3% of all mucosal melanomas, respectively (Chang *et al.*, 1998).

Patient Characteristics

Despite shared cellular origin, there are epidemiological differences between cutaneous and noncutaneous melanomas. Mucosal melanoma is commonly diagnosed in older individuals, with ~50% of all patients presenting at age >70 years; in contrast, only ~25% of all patients with cutaneous melanoma present at >70 years. Race and ethnic differences also exist: in the United States, <3% of cutaneous melanomas are diagnosed in African American and Hispanic populations; however, these ethnic groups present with 9% of all mucosal melanomas. Women are more likely than men to be diagnosed with anorectal melanomas (male-to-female rate ratio 0.75, 95% CI = 0.57–0.90) (McLaughlin *et al.*, 2005). It is unclear whether this female predilection is related to differences in biology, or to the fact that women are more likely to undergo perineal examination as part of their routine health care.

Sixty-five percent of diagnosed anorectal melanomas are located in the anal canal or at the anal verge; however, in 35% of cases disease is also identified in the distal rectum (Cagir *et al.*, 1999). Thus, it is not surprising that patients typically present with bleeding, anorectal discomfort, an appreciable mass, or change in bowel habits (See Figure 29.1). Diagnosis is often delayed because, as noted above, identifiable lesions are pigmented in only one third of cases; additionally, many believe that the disease itself is characterized by an aggressive biology. Therefore, at the time of correct diagnosis a majority of patients have advanced lesions measuring more than 2 mm in thickness (Wanebo *et al.*, 1981). Occasionally, melanotic lesions are found incidentally during pathologic examination of a hemorrhoidectomy or anal polyp specimen.

As noted above, noncutaneous melanomas are more likely than cutaneous melanomas to be diagnosed at an advanced stage. The incidence of locoregional lymph node metastasis at initial presentation is 61% for anorectal melanoma, 21%

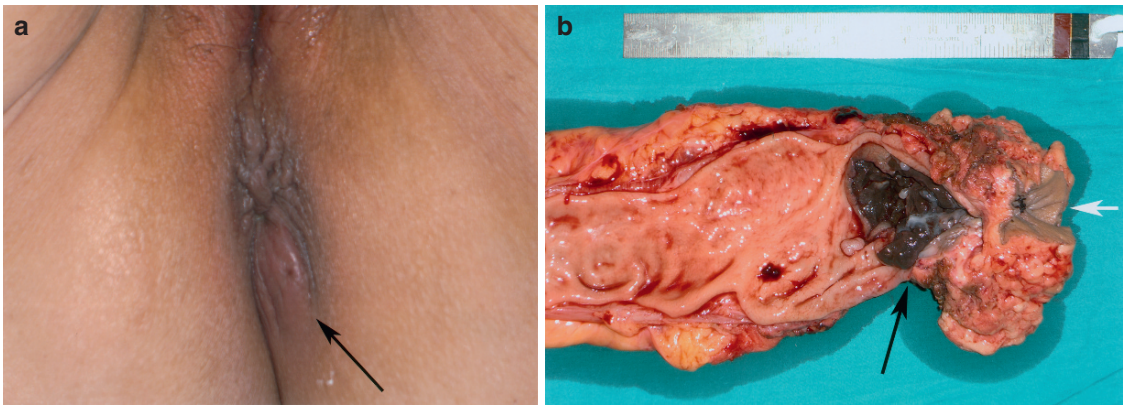

FIGURE 29.1. **(a)** Anal canal melanoma in a female patient presenting as a painful hemorrhoid (black arrow); **(b)** A bisected abdominoperineal resection specimen from the same patient showing large pigmented anal canal melanoma (black arrow) and resected anus (white arrow). (Photographs courtesy of Dr. Choong-Leong Tang, Singapore General Hospital, Singapore.)

for head and neck melanoma, 23% for melanoma of the female genital tract, and 11% for urinary tract melanoma; however, only 9% of cutaneous melanomas have lymph node metastases on presentation. At the time of diagnosis, distant metastases are identified in up to 29% of patients (Chang *et al*., 1998).

Risk Factors

In the United States, race is a strong predictor for development of melanoma. While rates of cutaneous and ocular melanoma are 5–20 times higher in Caucasians than in African Americans, the rate of mucosal melanoma is only twice as high. These findings suggest that cutaneous and ocular melanomas may share a common environmental risk factor (i.e., ultraviolet radiation) that does not pertain to mucosal melanomas. Sun exposure is not a risk factor for noncutaneous mucosal melanoma, and individuals with darker skin pigmentation have a higher incidence of anorectal melanoma. Some indirect evidence suggests that infection with the human immunodeficiency virus (HIV) may increase the risk of anorectal melanoma in young males (Cagir *et al*., 1999).

MOLECULAR PATHOGENESIS: KIT, BRAF, NRAS

The *KIT* receptor tyrosine kinase is crucial to normal development and function of melanocytes during the embryonic stage as well as postnatally. Loss-of-function mutations in *KIT* and its ligand results in white spotting phenotype in mice and humans; whereas activating *KIT* mutations are implicated in the pathogenesis of leukemia and gastrointestinal stromal tumors (GISTs) as well as some other human malignancies. Recently, *KIT* aberrations have also been associated with the pathogenesis of malignant melanoma.

The role of *KIT* in melanoma is complex. *KIT* expression is typically found in normal melanocytes (benign nevi and *in situ* melanomas) but appears to be downregulated in the invasive and metastatic melanomas. This progressive decrease in *KIT* immunopositivity is associated with increasing dermal invasiveness. One hypothesis is that *KIT* signaling impacts regulation of cell differentiation and tissue morphogenesis; therefore, the course of tumor progression may necessitate loss of its expression. In this context, reports of activating *KIT* mutations (and protein over-expression) in a subset of melanoma patients suggest a different mechanism through which the constitutive activation of *KIT*, through oncogenic mutations, promotes mitogenesis causing neoplastic transformation.

The genetic alterations associated with different sites of melanoma and varying levels of sun exposure indicate the existence of distinct molecular pathways and subsets. This significantly impacts on any molecular treatment of the disease. For example, *BRAF* mutations are most often seen in melanomas arising in the areas of skin that are intermittently exposed to the sun; however, these mutations are rare in skin that is chronically sun-damaged, or in never-exposed areas (acral and mucosal membranes) (Curtin *et al*., 2005). *NRAS* mutations occur only in melanomas without *BRAF* mutations, and do not appear to be associated with a specific clinical phenotype (Curtin *et al*. 2005). In contrast, increased copy numbers of *CCND1*

(Cyclin D1) or *CDK4*, two downstream genes in the Ras/MAPK signaling pathway, are seen in melanomas associated with chronic sun damage as well as in acral and mucosal melanomas, inversely correlating with *BRAF* mutations. This recently delineated genetic classification of malignant melanomas, based on the degree of relationship to ultraviolet light exposure, may have important treatment implications as targeted therapies are developed and tested.

Specifically in anorectal melanoma, Antonescu *et al*. (2007) assayed for *BRAF*, *NRAS*, *KIT*, and *PDGFRA* mutations. Interestingly, a heterozygous *KIT* exon 11 L576P substitution was identified in 3 of 20 patients tested. The 3 *KIT* mutation-carrying tumors were strongly immunopositive for *KIT* protein (Figure 29.2), and no *KIT* mutations were identified in tumors with <4+ *KIT* immunostaining. *NRAS* mutation was identified in one tumor. No *BRAF* or *PDGFRA* mutations were identified in either *KIT*-positive or -negative anorectal melanomas.

These findings indicate that anorectal melanomas lack *BRAF* mutations and infrequently show *NRAS* mutations (5%), demonstrating instead a higher rate of *KIT*-activating mutations than are found in nonacral cutaneous melanomas. The overall incidence of *KIT* immunoreactivity in anorectal melanomas was 20%, defined by a strong and diffuse pattern of staining. A good correlation between *KIT* immunoreactivity and the presence of *KIT* gene alterations at the molecular level was identified, with a 75% rate of *KIT* mutations in anorectal melanomas expressing strong *KIT* protein by immunohistochemistry.

Although more cases need to be tested, true *KIT* gene amplification does not appear to be a common event in anorectal melanoma; however, a modestly increased copy number of the *KIT* gene is seen in up to one third of cases (Figure 29.3). *KIT*-activating mutations and increased copy number are not mutually exclusive. One tumor in our study, showing an increased *KIT* copy number with FISH, also had a *KIT* L576P substitution mutation. Another

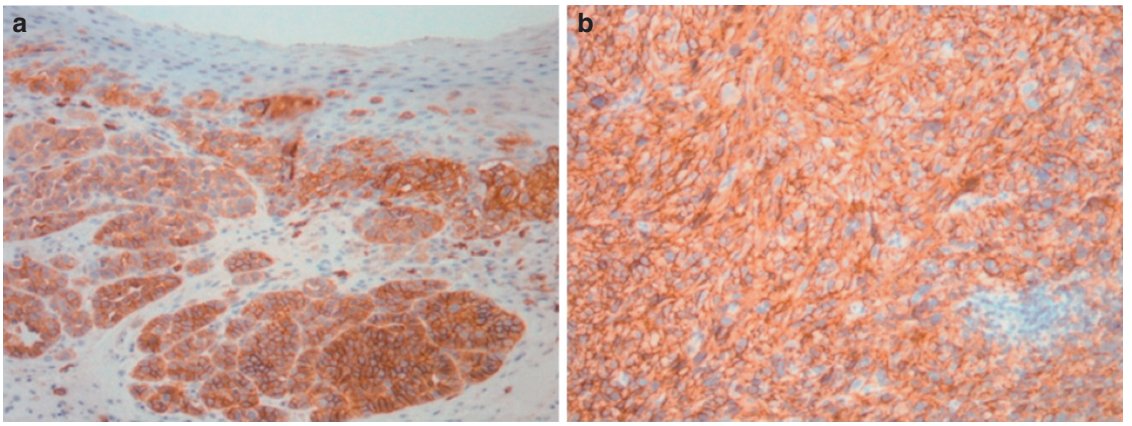

FIGURE 29.2. Immunohistochemical staining of **(a)** primary melanoma with strong and diffuse KIT (CD117) immunoreactivity in both intraepidermal (in situ) and invasive component (x100); **(b)** metastasis derived from the primary tumor, also strongly/diffusely positive for KIT (CD117) (x200). (From: Antonescu CR *et al*., 2007. *Used with permission of Wiley-Liss/Wiley*.)

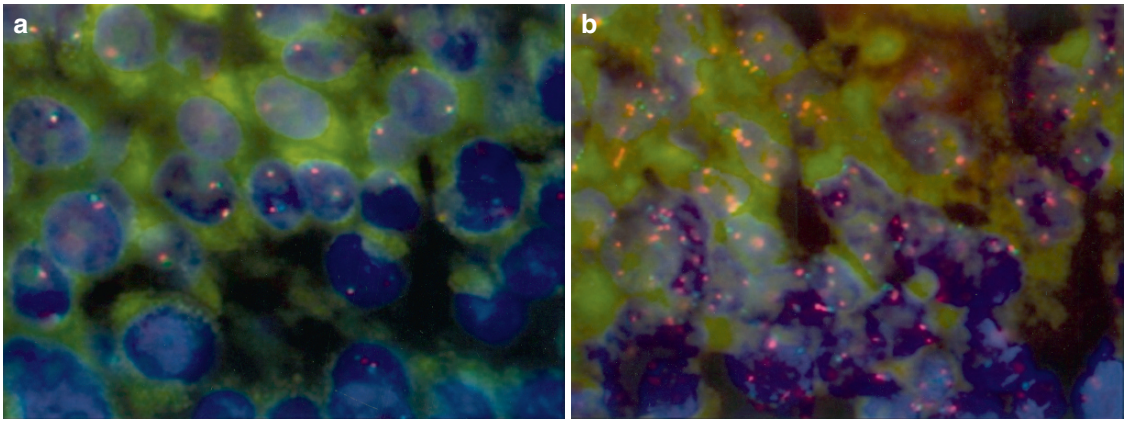

FIGURE 29.3. Fluorescence in situ hybridization showing (**a**) 2 copies of KIT (red) and centromere 4 (green) (Sample # 19); **(b)** increased copy number of KIT signals, with average ratio of KIT (red):centromere 4 (green) signals of 5:2 (Sample # 20). (From: Antonescu *et al*. 2007. Used with permission of Wiley-Liss/Wiley.)

important observation made by Antonescu *et al*. (2007) is that *KIT* protein phosphorylation can be seen in tumors with strong and diffuse *KIT* immuno-expression in the absence of identifiable *KIT/PDFGRA* mutations or increased copy number changes. This suggests that in these cases kinase inhibition might still be beneficial.

The findings correlate closely with results of earlier studies on the molecular phenotype of *KIT/PDGFRA* wild-type GIST tumors, which are also managed by specific *KIT* receptor tyrosine kinase inhibitors. The *KIT* L576P mutation in the juxtamembrane domain of *KIT* is seen not only in melanoma but has been described previously in GIST tumors (Antonescu *et al*., 2003). In a series of 500 GIST cases, Antonescu *et al*. (2005) reported that the incidence of L576P mutation was $<1\%$; these tumors were typically heterozygous and not associated with a specific clinical phenotype. In that series, only one of the L576P mutated GIST patients received imatinib for treatment of metastatic disease, developing secondary resistance to the drug after 13 months (Antonescu *et al*., 2005). Importantly, all of the *KIT* mutations reported to date for melanoma patients were somatic. No familial cases of melanoma or GIST were identified in association with this particular juxtamembrane mutation.

Antonescu *et al*. (2007) also performed drug testing in these mutants, revealing that the *KIT* L576P mutation is most sensitive to the dual SRC/ABL kinase inhibitor dasatinib. Dasatinib is orally active and small molecule ATP-competitive, which effectively inhibits the cell proliferation of nearly all imatinib-resistant BCR-ABL isoforms (Shah *et al*., 2004). Due to the structural homology of *KIT* activation loop mutants to imatinib-resistant BCR-ABL isoforms, dasatinib has been shown to inhibit *KIT* kinase activity, including in imatinib-resistant *KIT* activation loop mutants (Schittenhelm *et al*., 2006).

The *KIT* L576P mutant was also sensitive to imatinib and nilotinib, but required at least a tenfold higher dose than the imatinib-sensitive *KIT* V559D mutation.

The genetic classification of melanomas mentioned above incorporates anatomic location and ultraviolet light exposure, and has therapeutic implications (Curtin *et al.,* 2005). The most common type of melanoma, occurring on skin without chronic sun damage, frequently harbors either a mutation in *BRAF* or *NRAS*, suggesting a possible therapeutic intervention targeting the RAS-RAF-ERK and PI3K pathways. In contrast, melanomas on skin with chronic sun damage show frequent increases in the copy number of *CCND1*, and are likely to be less responsive to *BRAF* inhibitors such as sorafenib. The incidence of activating *KIT* mutations might also depend on the degree of sun exposure, varying from 2% in a study by Willmore-Payne *et al*., (2005) (which did not include any mucosal cases) to 13%. To this we can add the findings of Antonescu *et al*. (2007) that anorectal melanoma shows an increased prevalence of activating *KIT* mutation. Patients with *KIT* L576P mutant may be candidates for dasatinib therapy. Additional studies are needed to further develop this scheme and determine whether other subtypes of mucosal and acral melanomas not associated with ultraviolet light exposure demonstrate an increased incidence of *KIT* activation.

TREATMENT OF ANORECTAL MELANOMA

Although great strides have been made in identifying the molecular pathogenesis of anorectal melanoma in an attempt to develop new therapies, surgery is still the mainstay of treatment. The role of adjuvant treatments (chemotherapy, immunotherapy) is as yet unresolved. Surgical issues such as extent of surgery (radical versus local) and extent of lymphadenectomy remain controversial.

Extent of Surgical Resection

There is debate in the literature regarding the extent of surgery necessary for treatment of primary anorectal melanoma. Early studies suggested that aggressive treatment of the primary anorectal melanoma lesion with abdominoperineal resection was associated with improved outcome, possibly due to regional lymphadenectomy (Brady *et al*., 1995). However, other studies reporting on local excision of the primary anorectal lesion without regional lymphadenectomy described similar patterns of recurrence and survival, and no significant increase in local failure (Ward *et al*., 1986; Thibault *et al*., 1997). All studies have concluded that relapse is usually distant and lethal.

Because of the rarity of the disease, small retrospective studies provide the only guidance for treatment planning. The benefits of local excision are clear, including speedier recovery from a less invasive procedure, minimal impact on bowel function, and avoidance of colostomy. However, the local approach does not address the issue of regional lymph node metastasis, which is one of the most important predictors of outcome in primary cutaneous melanoma. This issue was addressed in an early series of 56 patients with localized anorectal melanoma, treated at Memorial Sloan-Kettering Cancer Center by either abdominoperineal resection or local excision between 1929 and 1993. This study

indicated a possible advantage of regional lymph node resection, as nine of ten long-term survivors were in the radical surgery group and had undergone abdominoperineal resection (Brady *et al*., 1995). In particular, two long-term survivors, one in the abdominoperineal resection group and one in the local excision group (who subsequently underwent therapeutic pelvic lymphadenectomy), had positive mesenteric lymph nodes. However, it is worth noting that the rate of isolated local recurrence was comparable in patients undergoing local excision and patients undergoing abdominoperineal resection. Additional reported series on local excision for anorectal melanoma have not reported high rates of isolated regional relapse, leading to the hypothesis that regional relapse is not the cause of patient demise (Bullard *et al*., 2003).

Reporting a more recent series from Memorial Sloan-Kettering Cancer Center, Yeh *et al.,* (2006) highlighted an alteration in practice patterns over time. In their study of 46 patients with anorectal melanoma analyzed over a period of 20 years, the authors noted a dramatic shift in treatment: from radical surgery such as abdominoperineal resection to more local treatment such as local excision (Table 29.1). These authors reported that, from 1984 to 1996, 21 patients were treated for primary anorectal melanoma and 15 of these (71%) underwent abdominoperineal resection. From 1997 to 2003, however, 25 patients were treated for anorectal melanoma and 21 of them (84%) underwent local excision. There was no change in patient demographics or in thickness or diameter of the tumors during these time periods. Patients who underwent abdominoperineal resection tended to present with lesions of greater thickness than those undergoing local excision: Median tumor thickness in the abdominoperineal resection group was 11 mm (range, 1.1–26 mm), compared with 7.2 mm (range, 1.1–19 mm) in the local excision group, but this difference was not statistically significant. Interestingly, however, despite the clear change in practice patterns during

TABLE 29.1 Primary tumor, treatment, outcome in patients treated before and after 1997. (From: Yeh *et al*. 2006. *Used with permission of Lippincott Williams & Wilkins*.)

	Total (n = 46)	1984–1996 (n = 21)	1997–2003 (n = 25)	*P*
Surgery				<0.0001
APR	19 (41%)	15 (71%)	4 (16%)	
LE	27 (59%)	6 (29%)	21 (84%)	
Thickness				NS
≤10 mm	30 (70%)	11 (58%)	19 (79%)	
>10 mm	13 (30%)	8 (42%)	5 (21%)	
First site of relapse				
All sites	34 (74%)	16 (76%)	18 (72%)	NS
Locoregional	12 (26%)	7 (33%)	5 (20%)	
Distant	13 (28%)	6 (29%)	7 (28%)	
Both	9 (20%)	3 (14%)	6 (24%)	NS

NS, indicates not significant.

these two time periods, the outcome was identical (Figure 29.4): 75% of patients recurred in each time period.

Yeh *et al*. (2006) also analyzed outcome based on extent of surgery. Five-year disease-specific survival for the entire cohort was 34%, with median follow-up of 39 months for survivors. Thirty-four of 46 patients relapsed within 10 months, with a recurrence rate of 53% at 1 year. As noted, the majority of patients developed distant recurrence. It is noteworthy that no difference was seen in relapse patterns between patients treated by abdominoperineal resection or those treated by local excision: five of 19 (26%) patients undergoing abdominoperineal resection and 7 of 27 (26%) undergoing local excision developed local recurrence as the first site of relapse. As discussed, survival was similar between both groups, with 5-year disease-specific survival of 32% and 35%, respectively, for the abdominoperineal resection cohort and the local excision cohort. These findings suggest that extent of resection is not associated with the rate of local recurrence or survival in anorectal melanoma.

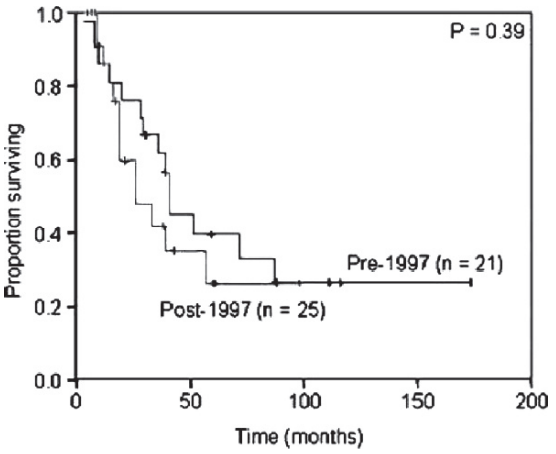

FIGURE 29.4. Changes in practice at Memorial Sloan-Kettering Cancer Center (MSKCC): Disease-specific survival before and after 1997. (From: Yeh *et al*. 2006. *Used with permission of Lippincott Williams & Wilkins*.)

Inguinal Lymphadenectomy

The need for regional lymphadenectomy in the surgical treatment of anorectal melanoma has also been debated. Inguinal, pelvic sidewall, and mesorectal lymph nodes are at risk for metastases from anorectal lesions. During abdominoperineal resection, mesorectal lymph nodes are resected *en bloc* with the primary tumor. Yeh *et al*. (2006) found that lymph node metastases did not predict outcome in patients undergoing abdominoperineal resection. It is likely that nodal disease in the setting of anorectal melanoma may not have the same biologic significance as nodal disease in the setting of cutaneous melanoma.

Prophylactic bilateral inguinal lymphadenectomy in patients without clinically palpable lymph nodes has fallen out of favor because it does not improve survival and carries a risk of complications. Elective inguinal dissection is recommended only in the setting of clinically apparent disease. Less invasive methods for identifying nodal disease, such as sentinel lymph node analysis, have been studied; however, in contrast to the relatively widespread acceptance of sentinel lymph node biopsy for extremity and truncal melanomas, the role of this procedure in anorectal melanoma has yet to be defined. This is partially due to uncertainly about whether or not nodal disease prognosticates or defines therapy.

As can be seen, the prognosis for anorectal melanoma is poor regardless of type of treatment. Many experts hypothesize that systemic dissemination is an early event in tumorigenesis and that, by the time the

lesion is clinically apparent, micrometastases are well established. Although there is no definite data supporting the use of adjuvant therapies, investigations continue and case reports provide hope for increased success in the future (Yeh *et al*., 2005).

Adjuvant Therapy

Chemotherapy

Cytotoxic chemotherapy is generally not effective in patients with cutaneous melanoma. Dacarbazine remains the most widely used single agent and is associated with a response rate of ~20%. However, the vast majority of responses are only partial, and median response duration is only 4–6 months. There are no phase III trial data to support a survival benefit for dacarbazine versus best supportive care/placebo control (Crosby *et al*., 2000). Temozolomide, an analog of dacarbazine, appears to have similar activity, is available in oral form, and may be associated with a lower frequency of central nervous system relapse.

Dacarbazine or temozolomide use in combination with a variety of other agents has not been found to have any advantage over single agent chemotherapy in large phase III trials. In one multicenter trial, 240 patients were randomly assigned to either dacarbazine or the four-drug combination known as the Dartmouth regimen (dacarbazine, cisplatin, carmustine, and tamoxifen). The Dartmouth regimen demonstrated a marginally better response rate (19%) than that of dacarbazine alone (10%), but the difference was not statistically significant: both treatment arms had a median survival of only 7 months. Bone marrow suppression, nausea/vomiting, and fatigue were more severe for patients receiving the combination therapy (Chapman *et al*., 1999).

Immunotherapy

Melanoma is considered more susceptible to immune attack than other tumor types, and has therefore been the tumor most studied as a target for immunotherapy. Melanomas are typically infiltrated with lymphocytes; an absence of infiltrating lymphocytes is associated with poorer prognosis. The absence of a primary tumor in ~5% of patients with metastatic melanoma suggests that the primary melanoma may have undergone an immune-mediated regression. A minority of patients with metastatic disease responds to immune mediators such as interferon-alpha and interleukin-2, suggesting that even metastatic melanoma is susceptible to immune attack. Additionally, primary melanoma is more common in immunosuppressed patients, who have an increased risk of developing other cancer types as well. Melanoma cells can be adapted to *in vitro* growth with relative ease, leading to laboratory investigations, identification of tumor antigens, and the development of immunotherapies such as monoclonal antibodies and T-cells against these antigens. These developments have resulted in the evolution of a large number of different immunotherapeutic strategies targeting melanoma, including antibodies and vaccines as well as interferon-alpha and interleukin-2. These will be discussed at greater length in other chapters in this series.

Radiation Therapy

Historically, melanoma has been considered a relatively radioresistant tumor, although newer data have challenged this

viewpoint. *In vitro* studies on melanoma cell lines have demonstrated widely differing radiation sensitivities (Rofstad, 1986), raising the possibility that some melanoma cells exhibit a type of radioresponsiveness similar to that shown by "late-reacting" or slow-renewal normal tissues, requiring greater-than-standard doses per radiation fraction for most effective cell killing (Bentzen *et al*., 1989). Radiation therapy is now being more commonly considered as a component in the therapeutic armamentarium.

A benefit for adjuvant radiotherapy following local excision was reported in a recent study from M.D. Anderson Cancer Center, in which 23 patients with anorectal melanoma underwent local excision (including lymph node dissection for patients with documented regional nodal disease), followed by adjuvant radiation therapy (Ballo *et al*., 2002). Radiation was delivered using a hypofractionated regimen of 30 Gy in 5 fractions over 2.5 weeks. Nine patients subsequently received adjuvant systemic therapy. The actuarial 5-year local control rate was 74%, although the actuarial 5-year disease-specific survival rate was only 36%. One fact not in doubt is that radiotherapy can provide effective palliation for the 40–50% of patients who develop unresectable local recurrence or metastatic disease producing bone pain, epidural spinal cord compression, central nervous system dysfunction due to brain involvement, and/or tumor hemorrhage.

PROGNOSIS

When stratified by anatomic sites, cutaneous melanoma has the highest 5-year survival rate of all melanomas (80%), followed by ocular melanoma (75%) and anorectal mucosal melanoma (20%). As discussed earlier, the generally poorer prognosis associated with anorectal melanoma may be caused by delay in diagnosis, by an inherently more aggressive behavior characteristic of this tumor, or by earlier dissemination of disease associated with the rich lymphatic and vascular supply of the mucosa.

Data reported by Yeh *et al*. (2006) in their study of 46 patients undergoing surgical resection of anorectal melanoma, with a median follow-up of 39 months, showed a 5-year disease-specific survival of 34%. Thirty-four of the 46 patients relapsed within a median of 10 months, with a recurrence rate of 53% at 1 year. The majority developed distant recurrence.

The strongest predictor of outcome in that series was the presence of perineural invasion in the primary tumor (Figure 29.5). All of the eight patients with perineural invasion recurred, compared with 67% of the 29 patients without perineural invasion. Median survival for the cohort with tumor perineural invasion was 19 months versus 39 months for the cohort without tumor perineural invasion. At last follow-up, only one of eight patients with perineural invasion was alive with lung, liver, and perirectal lymph node metastases, compared with 10 of 29 patients without tumor perineural invasion who were alive with no evidence of disease.

Tumor perineural invasion was the only independent predictor of disease-specific survival on multivariate analysis using Cox regression, with a hazard ratio of 3.4 (95% confidence interval 1.2–9.9, $p = 0.02$; Table 29.2). The presence of regional nodal metastasis, tumor thickness, and size were not associated with recurrence or survival

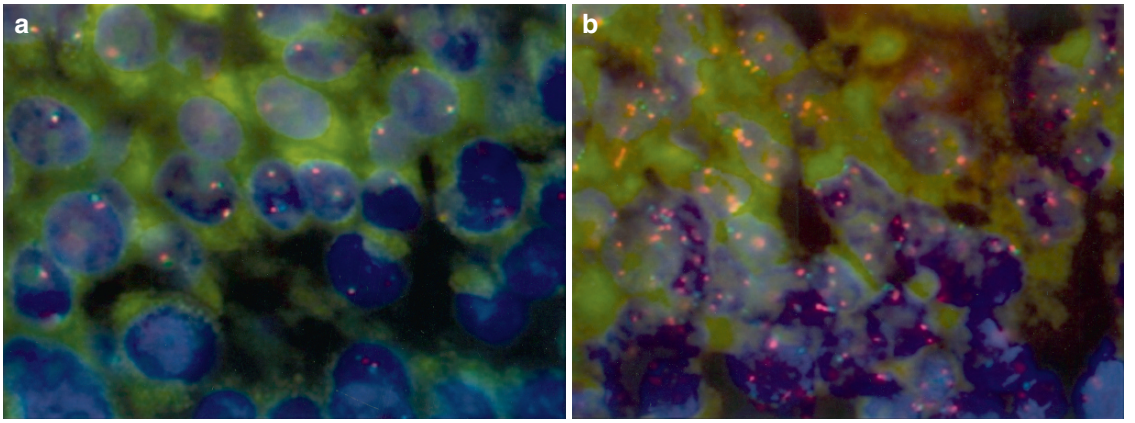

FIGURE 29.5. H&E stained histologic section of an anorectal melanoma showing epithelioid tumor cells in a nested growth pattern. Note presence of perineural invasion (arrow) where tumor cells grow tightly around a peripheral nerve branch. (From: Yeh *et al*. 2006. *Used with permission of Lippincott Williams & Wilkins*.)

TABLE 29.2. Multivariate analysis of prognostic factors associated with survival. (From: Yeh *et al*. 2006. *Used with permission of Lippincott Williams & Wilkins*.)

Variable	Hazard ratio	*P*
PNI	3.4 [1.2, 9.9]	0.01
Symptoms		0.62
Thickness		0.08
Diameter		0.23
Mural involvement		0.33
Necrosis		0.96

in this series of patients undergoing either abdominoperineal resection or local excision. Because anorectal melanomas are rare, staging has previously been limited to local, regional, and distant disease. The presence of perineural invasion, however, is an important independent prognostic factor, and should be considered in future clinical studies.

In conclusion, anorectal melanoma is a rare disease with a very poor prognosis, even when identified at an apparently early clinical stage, without evidence of distant disease. In view of the lack of evidence supporting more extensive procedures, local excision is the recommended treatment whenever technically possible. The aim is to minimize morbidity and maximize quality of life. Anorectal mucosal melanoma is molecularly different from the cutaneous form of the disease; *BRAF* and *NRAS* are rare. However, the new finding that *KIT* is overexpressed and mutated in a subset of patients with anorectal melanoma holds hope for the development of new molecularly based therapeutic strategies.

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