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Pierre Busson *Editor*

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NASOPHARYNGEAL CARCINOMA: KEYS FOR TRANSLATIONAL MEDICINE AND BIOLOGY
Pierre Busson

Nasopharyngeal Carcinoma

Keys for Translational Medicine and Biology

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DEDICATION

To my wife, Hélène, for her love, unflagging support and tireless dedication

PREFACE

Although listed as a rare cancer in a majority of countries, nasopharyngeal carcinoma (NPC) has a high incidence and appears as a serious public health problem in several highly populated areas of Southeast Asia and to a lesser extent in North Africa. Worldwide, its incidence rates at 85,000 new cases per year (Globocan 2008). Thus, in terms of incidence, among human virus-associated cancers, NPC ranks third behind liver and cervix carcinoma (750,000 and 530,000, respectively). In 2008, the mortality rate of NPC was still about 51,000 per year worldwide (Globocan).

NPC is a multifactorial disease related to dietary, viral and genetic factors. Despite fascinating biological characteristics, NPC has long been outside from the mainstream of cancer research for both scientific and socio-economical reasons. It is noteworthy that biological investigations have long been hampered by the paucity of laboratory models and the lack of data on premalignant states. However, recently, in several high incidence countries, strong economic growth and rapid progress in health care has propelled NPC onto the front stage of cancer research, worldwide. Simultaneously, in other places, the identification of head and neck cancers associated to human papilloma viruses has renewed interest for NPC as a proxy model of viral carcinogenesis.

As explained in the title, this book intends to contribute to "translational medicine and biology". By this, we mean a bi-directional process whose aim is to develop knowledge from basic science towards diagnostic and therapeutic applications and reciprocally to raise new questions for basic scientists. One general requirement for translational research is to establish a multidisciplinary knowledge base shared by the actors of various specialties. This is precisely the aim of the 12 chapters of this book. It will be useful for scientists, including PhD students, who want to become more familiar with the main concepts of NPC pathology, medical imaging and current therapeutics. Conversely, medical doctors who want to update their knowledge of NPC biology will benefit from chapters on viral and cellular oncogenesis and various aspects of host-tumor interactions.

Chapters 1 and 2 present the main concepts used for clinical and histological identification and classification of NPCs. Following this introductory part, we move to Chapter 3 dealing with all aspects of NPC epidemiology in a concise and comprehensive manner. Chapters 4, 5 and 6 review most aspects of NPC oncogenesis including virus/

cell interactions, genetic and epigenetic alterations and cellular interactions. Then we move back closer to clinical concerns with Chapters 7 and 8 that focus on biomarkers and medical imaging. The last four chapters are dedicated to therapeutics. Chapter 9 deals with the main concepts of NPC radiotherapy in terms accessible for the laymen. Chapter 10 provides an integrated view of all aspects of the medical or systemic treatments of NPC. Chapter 11 focuses on NPC immunotherapy which is currently a very dynamic field. The last chapter on therapeutic induction of apoptosis opens perspectives for novel targeted therapies of NPC.

Finally I want to acknowledge all authors who did their best to go beyond their own research interest and provide a comprehensive view of their subject. However many aspects remain to be improved. All remarks and suggestions from the readers will be welcomed (pbusson@igr.fr).

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PIERRE BUSSON is a Senior Research Scientist at the CNRS (National Center for Scientific Research) and the Gustave Roussy Institute in Villejuif, close to Paris. He obtained his MD in 1983 and his PhD in Virology in 1990 from Paris University. Following a post-doctoral research fellowship at the Lineberger Cancer Research Center (Chapel Hill, North Carolina, USA), he was recruited by the CNRS in 1992 and established his own group in the Gustave Roussy Institute in 2001.

His major research interests are cellular interactions in Epstein-Barr virus-associated nasopharyngeal carcinomas, with special emphasis on the role of tumor exosomes and other secreted nano-objects, in a perspective of translational research. Recently, his group has extended the same type of approach to ovarian carcinomas. He published about 75 articles in peer-reviewed scientific journals.

Dr Pierre Busson has been member of the Board of the International Association for Epstein-Barr virus research from 2000 to 2004. He has been actively involved in the organization of bi-annual international meetings on nasopharyngeal carcinomas (2003, Villejuif; 2005, Toronto; 2007, Brisbane; 2009, Marrakech; 2011, Penang). He is a grant reviewer for several institutions in France, Europe and in Hong-Kong. He is academic editor for PLoS One and reviewer for about 20 journals.

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CHAPTER 1

DIAGNOSIS AND CLINICAL EVALUATION OF NASOPHARYNGEAL CARCINOMA

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Abstract: Nasopharyngeal carcinoma (NPC) is an epithelial malignant tumor which arises from the mucosa of the nasopharyngeal cavity. NPC usually presents as painless neck lumps. It can also present with nasal, aural and/or opthalmo-neurologic symptoms. Patients in early stage of the disease are often asymptomatic or present with apparently trivial symptoms. Diagnosis is based on histopathological examination of the biopsied tissue obtained through endoscopy of the nasopharynx. Delayed diagnosis remains a problem in NPC. The most common used staging system is the "tumor node metastasis (TNM)" system, jointly developed by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC).

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a unique entity among carcinomas of the upper respiratory and digestive tract, with distinct geographical distribution[1,2] and is strongly associated with Epstein Barr Virus.[3] The nasopharynx is the uppermost part of the pharynx that lies behind the nasal cavity (post nasal space). The Fossa of Rosenmüller (FOR) of the nasopharynx is the most common site of origin of NPC.[4,5] The tumor spreads anteriorly into the nasal cavity, inferiorly into the oropharynx, superiorly into the skull base, laterally into the parapharyngeal space and posteriorly into the retropharyngeal space (Fig. 1). When it spreads into the skull base, it leads to compression of cranial nerves. NPC commonly spreads by lymphatics to the cervical lymph nodes. Lymphatics of the FOR drain into the node of Rouvier within retropharyngeal space and subsequently to

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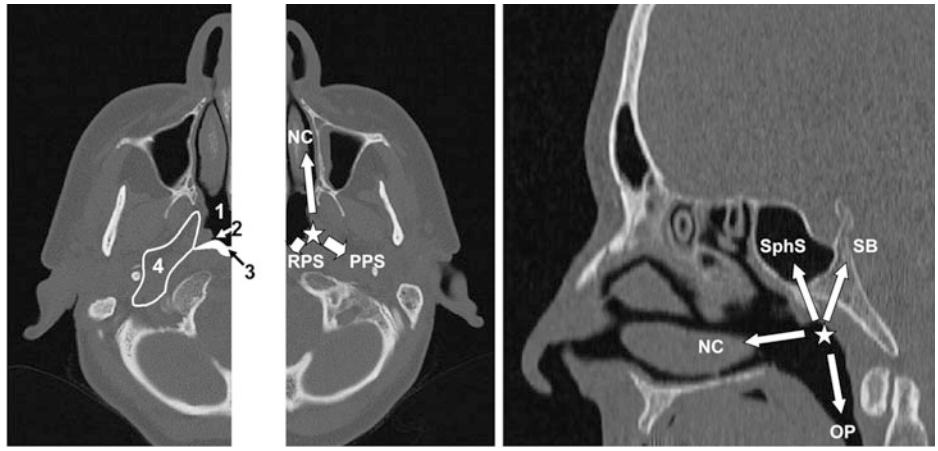

Figure 1. Anatomical relationship of the nasopharynx and preferential routes of local tumor spread. 1: nasopharynx; 2: fossa of Rosenmüller; 3: retropharyngeal space; 4: parapharyngeal space. NC: nasal cavity; RPS: retropharyngeal space; PPS: parapharyngeal space; SphS: sphenoid sinus; SB: skull base; OP: oropharynx; *: most common initial tumor site.

the upper deep cervical lymph nodes.[6] This explains why the neck lump is often the first presenting symptoms of NPC. NPC may spread through the blood stream (hematogenous route) to distant sites such as the bone, lung and liver.

CLINICAL DIAGNOSIS OF NPC

Clinicians need to be aware that NPC patients often present with nonspecific symptoms and signs in the head and neck region. A proper clinical workup begins with a detailed history of the presenting complaints. The next step is a thorough physical examination including endoscopic examination of the head and neck region. This should be followed by investigations to confirm the diagnosis and assess the extent of the disease prior to treatment.

Among cases of newly diagnosed NPC (totalling over 1200 cases), reported from the year 2007 to 2010 to the Malaysian Nasopharyngeal Carcinoma Database,[a] the most common presenting symptoms were neck lumps (40%), nasal symptoms (blood stained nasal discharge, blood stained saliva, or nasal blockage) (26%), aural symptoms (unilateral blocked ear, pressure sensation in the ears, mild hearing loss or tinnitus) (14%) and ophthalmo-neurologic symptoms (unilateral facial numbness, diplopia or unilateral headache) (10%). Similar spectrum of presenting symptoms are reported elsewhere in the world across time.[7-10]

[a] The Malaysian Nasopharyngeal Carcinoma Database (http://app.acrm.org.my/npc) was funded by the Ministry of Health Malaysia and set up by the Malaysian Nasopharyngeal Carcinoma Study Group, which comprise of a network of institutes including Penang Hospital, Kuala Lumpur Hospital, Queen Elizabeth Hospital, Sarawak General Hospital, University of Malaya Medical Centre, University Science Malaysia Hospital, Institute for Medical Research and Cancer Research Initiatives Foundation (CARIF).

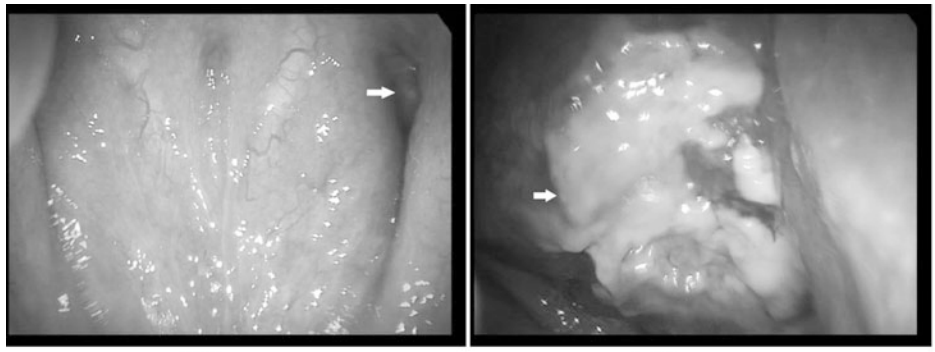

Figure 2. Nasopharynx as viewed through a fiberoptic endoscope. i) Normal nasopharynx showing the fossa of Rosenmüller (arrow). ii) Nasopharyngeal carcinoma (arrow).

As the nasopharynx is located in a confined space behind the nasal cavity, examination of this area is usually carried out using a flexible fiberoptic or rigid endoscope. The endoscope is connected to a camera system to allow the operator a close view of the nasopharynx. The NPC may appear as a mass in the nasopharynx (Fig. 2). Biospies are usually taken from this area to confirm the diagnosis. In certain instances, the nasopharygeal mucosa may appear normal although the tumor might be present under the mucosa (submucosal tumor). Magnetic resonance imaging (MRI) is useful to identify these submucosal tumors and to serve as a guide for biopsy.

The diagnosis of NPC is usually achieved by histopathological examination of the biopsied specimens. NPC is classified as keratinizing or nonkeratinizing (Table 1), the latter being the predominant type of NPC in endemic areas.[10] On the basis of electron microscopy, the NPC types are regarded as variants of squamous cell carcinomas (see Chapter 2 by Nicholls and Niedobitek).[11,12] This includes the undifferentiated type, which have morphological characteristics of undifferentiated epithelial cells upon examination by light microscopy.[11,13]

Table 1. WHO histopathological classification of NPC[10,16]

WHO Classification (2005)	Former Terminology (WHO 1998)
Keratinizing carcinoma	WHO Type 1
Nonkeratinizing carcinoma	
- differentiated	WHO Type 2
- undifferentiated	WHO Type 3
Basaloid squamous cell carcinoma	(no former terminology)

Adapted from reference 10 (Barnes L et al, eds. World Health Organization Classification of Tumours. Pathology and Genetics of Head and Neck Tumours Vol 9. Lyon, France: IARC Press 2005; 85-97); and from reference 16 (AJCC Cancer Staging Manual, 7th ed, (2010) published by Springer Science and Business Media LLC, www.springer.com. Used with the permission of the American Joint Committee on Cancer (AJCC), Chicago, Illinois).

Since NPC has a high propensity to spread to the regional lymph nodes, it is imperative to examine the neck region to detect involvement of the cervical lymph nodes. Fine needle aspiration cytology of the enlarged cervical lymph nodes can be performed to confirm nodal involvement.

As NPC may cause cranial nerve palsies, the cranial nerves should be examined. The patient should also be examined for distant metastasis to the bones, lungs and liver. The local and distant spread of the disease, are confirmed using diagnostic imaging modalities.

STAGING

Staging is a universal language used within the medical profession to communicate information about the extent of any cancer. This step is very important in order to make comparisons, to determine the prognosis and to assist in overall decisions on the choice of treatment modalities.

Staging may be carried out at several points during the care of the patient. Of these, the clinical stage (pretreatment stage) is the commonly used point. Clinical staging incorporates information obtained from symptoms, physical examination, endoscopic examinations as well as imaging of the tumor, regional lymph nodes and metastases.[14] It includes any information obtained about the extent of cancer before initiation of definitive treatment.[14]

Details of the presenting complaints would give an idea of the stage of the disease. Nasal and aural symptoms could be due to a tumour which is still confined to the primary site of the nasopharynx (i.e., may be as early as T1). The presence of a neck mass is a manifestation of disease spread to the cervical lymph nodes (N1-3) and is an indication that the tumor has already reached the next stage of spread to the regional lymph nodes. Ophthalmo-neurologic symptoms signify infiltration to the skull base (T4) which is considered as an advanced stage. The duration of the presenting complaints may give an idea of the aggressiveness of the tumor.

Staging is performed by clinical examination followed by imaging, such as, computerized tomography (CT), magnetic resonance imaging (MRI), chest X-ray, ultrasound, bone scintigraphy and ^{18}F-fluoro-2-deoxy-D-glucose positron emission tomography (PET) scans. The exact combination of imaging modalities used depends on their availability and cost and may differ from one centre to another. For clinical staging of NPC, the National Comprehensive Cancer Network (NCCN) guidelines suggests CT with contrast or MRI with gadolinium (covering the region from the skull base to the clavicles), PET/CT for Stage III-IV disease[15] (see Table 2) and PET or PET/CT for detection of distant metastasis (lung, liver, bone) for N2-3 disease.[15]

The “tumor node metastasis” (TNM) staging system, jointly developed by The American Joint Committee on Cancer (AJCC)[14,16] and the International Union Against Cancer (UICC), is the most commonly used system. This staging system is primarily based on anatomy, in which, T refers to the local extent of the primary tumor, N refers to the extent of regional nodes involvement and M refers to the distant spread (metastasis) of the tumor. The TNM scores are then combined to determine the overall stage[14] (Table 2).

Rarely, NPC may be detected as pre-invasive carcinoma in situ[17,18] (Tis, N0, M0). This is classified as Stage 0.[16]

As the imaging modalities differ in their sensitivity, the stage determined could also differ depending on the modalities used. If there exist uncertainty in classifying or staging the disease, the lower category will be used.[14] This also means that centers which use

Table 2. TNM clinical classification for tumors of the nasopharynx (AJCC Staging, 7th Edition)

Primary Tumor (T)

- T1—Tumor confined to nasopharynx, or extends to oropharynx and/or nasal cavity without parapharyngeal extension
- T2—Tumor with parapharyngeal extension (posterolateral infiltration of tumor)
- T3—Tumor involves bony structures and/or paranasal sinuses
- T4—Tumor with intracranial extension and/or involvement of cranial nerves, hypopharynx, orbit, or with extension to the infratemporal fossa/masticator space

Regional Lymph Nodes (N)

- N0—No regional lymph node metastasis
- N1—Unilateral metastasis in cervical lymph node(s), 6 cm or less in greatest dimension, above the supraclavicular fossa, and/or unilateral or bilateral, retropharyngeal lymph nodes, 6 cm or less, in greatest dimension
- N2—Bilateral metastasis in cervial lymph node(s), 6 cm or less in greatest dimension, above the supraclavicular fossa
- N3—Metastasis in a lymph node(s) greater than 6 cm and/or to supraclavicular fossa
- N3a—Greater than 6 cm in dimension
- N3b—Extension to the supraclavicular fossa

Distant Metastasis (M)

- M0—No distant metastasis
- M1—Distant metastasis

Clinical Stage Groups (Anatomic Stage/Prognostic Groups)

Stage I: T1, N0, M0

Stage II: T1, N1, M0; T2, N0, M0; T2, N1, M0

Stage III: T1, N2, M0; T2, N2, M0; T3, N0, M0; T3, N2, M0

Stage IVA: T4, N0, M0; T4, N1, M0; T4, N2, M0

Stage IVB: Any T, N3, M0

Stage IVC: Any T, any N, M1

Adapted from Edge SB, DR, Compton CC, Fritz AG, Greene FL, Trotti A, eds. AJCC Cancer Staging Manual. 7th ed. New York: Springer, 2010:44-46.[16] Used with the permission of the American Joint Committee on Cancer (AJCC), Chicago, Illinois. The original source for this material is the AJCC Cancer Staging Manual, Seventh Edition (2010) published by Springer Science and Business Media LLC, www.springer.com.

less sensitive imaging modalities may under-stage their patients. This should be borne in mind when evaluating studies on the stage of disease, and, is of particular relevance in areas which may not have the state of the art imaging modalities for staging.

The MRI currently provides the most sensitive and accurate evaluation of the primary tumor (T classification) and is preferable to CT for this purpose.[19] In comparison to CT, MRI was reported to be more precise in detecting the extent of the tumor, resulting in changes in the T classification for almost 50% of cases, as well as changes in the N classification and clinical stage in 11 and 39% of cases respectively.[20] As MRI is superior

to CT for soft tissue discrimination, it is able to differentiate retropharyngeal lymph node (RLN) metastasis from parapharyngeal extension of the primary tumor. This is important because the presence of RLN metastasis correlates with prognosis[21-23] and is now included in the AJCC Staging (7th Edition).[16]

While MRI is superior to PET/CT in demonstrating tumor invasion in the parapharyngeal space, base of the skull, intracranial area, sphenoid sinus and retropharyngeal lymph nodes, PET/CT is superior in demonstrating spread to the cervical lymph nodes.[24,25] PET/CT is the most sensitive, specific and accurate modality for detection of distant metastasis.[24-26] PET/CT has been reported to be superior, and able to replace conventional investigations such as chest radiography, abdominal ultrasound and skeletal scintigraphy for staging of distant metastases (M staging).[27] While MRI is superior to PET/CT to detect recurrence/residual disease at the primary site, combination of MRI and PET/CT is superior to either modality alone for restaging.[28]

The staging system undergoes periodic revisions in order to improve the classification of the extent of the tumor. In evaluating the AJCC Staging (6th Edition), Mao et al found that survival curves of the different T/N subsets showed a better segregation when Stage T2a was downstaged to T1, T2b and T3 were incorporated into T2, and the nodal greatest dimension was not used as a criteria for N staging.[29] In line with this, in the AJCC 7th Edition,[16] changes were made to staging of the cancer of the nasopharynx. T2a lesions is now designated T1. Stage IIA is now classified as Stage I. Lesions previously staged as T2b is designated as T2 and Stage IIB is now designated as Stage II. Retropharyngeal lymph node(s), regardless of unilateral or bilateral location, is now considered N1 in the AJCC Staging (7th Edition).[16] The criteria which was used in the AJCC Staging (7th Edition) was found to be superior to the AJCC Staging (6th Edition). Edition as the revised criteria provided better segregation of survival curves.[30]

It is important to take note of the version of staging when comparing cancers staged in studies at different time points. In some instances when comparing studies across time, it is possible that the apparent overall decrease in stage could be due to revisions of the staging criteria rather than actual differences in the extent of the cancer.

DELAYED DIAGNOSIS OF NPC

Delayed diagnosis remains a major issue in NPC.[7-9] Although NPC may be curable in the early stages, most patients present to the clinicians at late stages. In our series from the Malaysian Nasopharyngeal Carcinoma Database,[a] majority of cases (75%) presented at Stage III/IV. Symptoms such as blood stained nasal discharge, blood stained saliva and unilateral nasal/aural symptoms (such as unilateral nasal blockage, unilateral middle ear effusion symptoms such as blocked ear, pressure sensation in the ears, mild hearing loss or tinnitus) could be dismissed by patients and even doctors, as trivial, missing the chance of early diagnosis.[7]

Owing to the hidden location of the tumor and their indirect manifestations, diagnosis can be delayed for as much as six months in 70% of patients.[31] Up to 13% of patients may also present with neck lumps without a visible primary tumor (a situation known as ‘occult primary’).[32] Serial and multiple biopsies are sometimes necessary due to submucosal disease, and false negative histopathological examination. Fine-needle aspiration cytology of neck metastases at best has an accuracy of 82.6%.[33]

Table 3. ECOG* performance scale/Zubrod score[16,35]

ECOG Score	Performance	Karnofsky Score[34]
0	Fully active, able to carry on all predisease activities without restriction.	90-100
1	Restricted in physically strenuous activity but ambulatory and able to carry work of a light or sedentary nature.	70-80
2	Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50% of waking hours.	50-60
3	Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.	30-40
4	Completely disabled. Cannot carry on self-care. Totally confined to bed.	10-20
5	Death	0

*Eastern Co-operative Oncology Group, Robert Comis, M.D., Group Chair.
Adapted from Edge SB, DR, Compton CC, Fritz AG, Greene FL, Trotti A, eds. AJCC Cancer Staging Manual. 7th ed. New York: Springer, 2010:44. Used with the permission of the American Joint Committee on Cancer (AJCC), Chicago, Illinois. The original source for this material is the AJCC Cancer Staging Manual, Seventh Edition (2010) published by Springer Science and Business Media LLC, www.springer.com.

Currently, diagnosis is made by endoscopic examination followed by biopsy of the suspected tumor. This procedure requires skill and is usually carried out by ENT specialists. Diagnosis of NPC at an early stage would require detection of the tumor prior to the appearance of metastatic lymph nodes in the neck or other local extension.

Other than clinical staging, the overall health of the patient prior to treatment is evaluated using the Karnofsky General Performance Status[34] or Eastern Co-operative Oncology Group (ECOG) Performance Status, (also known as the WHO score or Zubrod Score).[16] The ECOG Performance Status is a set of scales and criteria used to assess how the disease affects the activities of daily living abilities of the patient. (Table 3).[35] In NPC, most patients have minimal impairment of performance status, even though they may be at a late stage of the disease. This lack of general symptoms and the feeling of general wellbeing further clouds the patient's decision to seek medical advice and delays the time of diagnosis.

RECURRENT NASOPHARYNGEAL CANCER

After completion of treatment for NPC, patients would need to be followed up and evaluated for the possibility of recurrence. The latency for recurrence vary widely.[36] Recurrence may occur within the nasopharynx (local recurrence), regional lymph nodes (regional/nodal recurrence) or at distant metastatic sites. The clinical workup is similar to that of the primary disease. Staging may be carried out prior to treatment of the recurrent tumor using the same classification with the r prefix (rTNM).[14]

It should also be noted that early recurrence of NPC could be due to geographical miss during radiotherapy, in which part of the cancer was not included in the irradiated volume. This may be clinically indistinguishable from true recurrence. Recurrence may have a long latency in NPC. In a series of over 800 patients with nasopharyngeal carcinoma, recurrence could occur even after 5 years in 9% of cases.[36] However, local recurrence of NPC (i.e., in the nasopharynx) may be clinically indistinguishable from newly formed radiation-induced tumor from the same site.

CONCLUSION

The most common presenting symptom of NPC is a neck lump. The neck lump is actually a regional metastatic lymph node, which, is a sign that the cancer had already spread. Nasal and aural symptoms, which may be present at the early stage of the disease, are trivial and may be disregarded by the patient or even the professionals, thus reducing the chance of early diagnosis. In addition, most NPC patients have minimal impairment of their general performance status. Definitive diagnosis requires endoscopic guided biopsy of the nasopharynx. All these pose major challenges to early diagnosis of NPC, especially if patients are to wait for significant clinical symptoms or poor general health before seeking treatment. Thus, screening procedures, which can be carried out on patients with trivial symptoms or even asymptomatic individuals, would be very helpful. Newer ways to predict the risk of recurrence are also eagerly awaited.

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CHAPTER 2

HISTOPATHOLOGICAL DIAGNOSIS OF NASOPHARYNGEAL CARCINOMA: Looking beyond the Blue Book

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Abstract: Nasopharyngeal carcinoma (NPC) is a malignant tumour of the nasopharynx that has a strong geographical distribution, with a high incidence in Southern China. It is a tumour that has had many classification schemes proposed since the early 20th century. The latest classification proposed by the World Health Organization has two main types of tumour—nonkeratinizing carcinoma and keratinizing squamous cell carcinoma. In actual practice, however, histological gradations between these two types can be present and the prognostic significance of such subdivision remains unclear. As there has been an increasing trend of monitoring NPC by Epstein-Barr viral (EBV) load it is possible that future classifications may be based on whether the tumour is associated with EBV or not, rather than histological appearances.

INTRODUCTION

The primary function of the pathological diagnosis of a nasopharyngeal biopsy from a patient with a suspected lesion is to provide a diagnosis for the clinician so as to enable both adequate treatment as well as some degree of prognostication to the patient for the diagnosis rendered. The nasopharynx is in one of the most hidden anatomical regions of the body and for such a small area it is surprising that there has been so much confusion and debate over the classification of nasopharyngeal carcinoma or NPC. This chapter will chronologically detail the expanding knowledge and concepts about NPC and explain how this tumour has defied accurate classification since the beginning of the

Nasopharyngeal Carcinoma: Keys for Translational Medicine and Biology,
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20th century. It will explain the rationale behind the current World Health Organization classification of NPC and detail the strengths as well as concerns over this classification.

The pathologist has a vital role to play in the diagnosis of NPC, both in the primary diagnosis and in the identification of tumour relapses. As NPC occurs in a worldwide distribution, the desirability of a universally satisfactory and acceptable classification scheme is obvious so that diagnosis can be standardized and that comparison of diagnostic, therapeutic and other studies can be comparable from investigators around the world.

For most pathologists there have been two series of publications available worldwide that have attempted to provide some degree of uniformity on the classification of tumours. The Armed Forces Institute of Pathology (AFIP) based in the United States of America has provided a series of fascicles and the International Agency for Research on Cancer (IARC) under the umbrella of World Health Organization (WHO) has also published another series which have colloquially been referred to as the 'blue books'. In 2005 the latest update to the latter series dealing with tumours of the head and neck was produced.[1] This chapter will also critically appraise the latest classification of NPC.

EARLY CLASSIFICATION SCHEMES FOR NPC

The development of a classification scheme for nasopharyngeal carcinoma has been attempted many times since the early 20th century. Until the early 1900s NPC was considered to be a rare tumour and references to this lesion were usually absent in textbooks of clinical medicine or surgery. Only since the late 1960s has the literature on this unique tumour elucidated its peculiar clinical, pathological, radiographic, epidemiologic, serologic and therapeutic features. These studies have shown that nasopharyngeal carcinoma is composed of several similar but morphologically different carcinomas, that certain types of this tumour have their highest incidence in populations of Oriental descent, that there are probable environmental factors of significance and that certain types of nasopharyngeal carcinoma are associated with Epstein-Barr virus (EBV).

In the early 1900s, the classification of malignant nasopharyngeal neoplasms was simple; a distinction was made only between those arising from the surface epithelium (epitheliomas) and lymphosarcomas arising from the submucous lymphoid tissue. The first report of NPC in the English literature was in 1901[2] with a detailed clinical series published ten years later[3] but a histological classification of the carcinomas was first attempted in 1903 by Citelli and Calamida (reviewed in ref. 4) who initially divided NPC into three groups: a mixed carcinoma, a pure cylindric carcinoma arising from glands and a pure squamous cell carcinoma. This classification was expanded by dividing the cylindric carcinomas into two different groups, carcinoma cylindrocellulare solidum and adenomatosum arising from the surface epithelium and glandular epithelium respectively (reviewed in ref. 4). The carcinomas were further subdivided in 1922 by Duval and Laccasagne into degrees of differentiation but most American and English authors adopted the Broders classification published later. The descriptive terms proposed by Bang (carcinoma basocellulare, carcinoma planocellulare mucous membrane type, paraketatoticum and cornescens) emphasized the descriptive limits to which the 4 stages of differentiation were being promoted (reviewed in ref. 4).

The lymphosarcomas were initally separated from the epitheliomas but later it was realized that the tumours called lymphosarcomas were actually lymph node metastases resulting from a primary tumour located in the nasopharynx. For instance, one of the

first studies in Chinese patients in 1923 documented 90 cases of cervical lymphosarcoma but later review considered these to be lymph node metastases of NPC. A new tumour with an intimate relation between epithelial cells and lymphoid tissue was reported in 1921 in two simultaneous works—one in France by Reverchon and Coutard, inspired by Regaud, the other in Germany by Schmincke, the Munich pathologist (reviewed in ref. 4). Both authors stressed the unique histological picture of the tumour and the unique sensitivity to radiotherapy. The tumour was called lymphoepithelioma as there was an intimate mixture of large polygonal cells and lymphoid cells in a syncytial character.

The Regaud morphology was defined as consisting of well circumscribed strands of epithelial cells with large, pale staining vesicular nuclei and poorly delineated cytoplasm, embedded in a stroma more or less rich in lymphocytes. The nuclei were round and nucleoli were prominent. No features of keratinization could be identified. Within the epithelial groups were small nuclei considered to be lymphocytes. Sometimes tumour cell nests were separated by a fibrous stroma.

The Schmincke type, in contrast, contained epithelial cells in irregular anastomosing trabeculae of ill-defined cells with large vesicular nuclei. In many places the appearance of the epithelial columns was lost and the cells became dissociated from one another giving rise to a reticular mass of round, oval or polygonal cells. All stages of transition could be found between the epithelial cells forming trabeculae and those in the syncytial type masses and it therefore seemed that the two classical types were not separate but actually merged into one another.

Only a few years after Regaud and Schmincke's publication another type of radiosensitive tumour was described by Quick and Cutler[5] which was called transitional cell carcinoma. They identified a group of patients with intraoral carcinomas which were both susceptible to radiation treatment and had a peculiar histological appearance—lacking the usual features of squamous cell carcinoma. These cells were small, uniform in size with a relatively large hyperchromatic nucleus and scanty cytoplasm, closely packed with little intercellular ground substance. The cells formed solid sheets, growing in anastomosing columns of opaque granular polyhedral cells with convolutions. Flat, pavement characteristics, spines, keratinization and pearl formation (indicative of squamous differentiation) were absent. The authors considered this condition to be markedly different from the routine squamous cell carcinoma of the intraoral region but only 2 of the cited cases were from the nasopharynx. Though the term 'transitional' referred to an epithelium not seen in the nasopharynx, the radiosensitivity of this tumour and of the cervical gland metastases[6] resulted in this tumour being associated more with the lymphoepithelioma (an entity of which apparently Quick and Cutler were unaware of) than squamous cell carcinoma. Later Cutler characterized the transitional cell carcinoma as a lymphoepithelioma without the lymphoid elements.

In 1929, Ewing addressed the problem of the lymphoepithelioma[7] and stated that an epidermoid carcinoma, or transitional cell carcinoma, when associated with lymphoid tissues (as in the case of the nasopharynx) became difficult to separate from lymphoepithelioma and that the separation of the latter tumour from the others remained a somewhat arbitrary decision. Cappell in 1934[8] and 1938[9] came to the same conclusion. The transitional cell carcinoma could be distinguished from the other two groups by a more obvious origin from the surface of the epithelium and the pattern of growth. The cells formed broad alveoli in which central necrosis and degeneration were more common. Yet again there was no trace of keratinization in that the cells were devoid of

intercellular bridges and squamous pearls. There was an overall absence of the intimate mixture of the lymphocytes with the tumour cells as seen in the 'lymphoepithelioma'. Even so, he as well as other pathologists of the time found difficulty in separating the transitional cell carcinoma from lymphoepithelioma. Even though Cappell thought that the lymphoepithelioma was more common than the transitional cell carcinoma he considered that there was great interobserver error. The place for transitional cell carcinoma in the 1940s still remained unclear—several workers claimed that it was inseparable from lymphoepithelioma whilst others saw it as a squamous cell carcinoma of low grade differentiation. Clearly there was also much variation between pathologists; the studies of Salinger and Pearlman[10] had a number of nasopharyngeal neoplasms judged microscopically by 3 independent pathologists, all of whom came to divergent results concerning the tumours of low grade differentiation.

The separation of nasopharyngeal carcinoma into the three elements lymphoepithelioma, transitional cell carcinoma and squamous cell carcinoma persisted into the 1950s. Thomson[11] reinforced previous observations in his studies on transitional cell carcinoma in that he documented the appearance of central necrosis and an origin from the surface epithelium. He also agreed that though attempts were made to separate transitional cell carcinoma from lymphoepithelioma and lymphosarcoma it was difficult. He considered that intercellular bridges were useful for the diagnosis of epidermoid carcinoma and silver stains for reticulin were helpful for separating lymphoepithelioma from lymphosarcoma. To highlight the difficulty in diagnosis he described one patient who had a diagnosis of lymphosarcoma made which was then changed to epidermoid carcinoma, malignant atypical epithelial cells and finally lymphoepithelioma.

Within the 1930s and 1940s the high frequency of NPC in Chinese people became more evident. Originally 90 cases of 'cervical lymphosarcoma' were described in 1923 but these were actually considered on further review to be lymph node metastases of nasopharyngeal carcinoma (reviewed in ref. 4). In 1935 Ch'eng[12] described 7 clinical cases of lymphoepithelioma and in 1940 Ch'in and Szutu 90 cases.[13] Professor Digby from Hong Kong published 240 cases of nasopharyngeal carcinoma occurring in the Chinese of Hong Kong and noted that there was a male predominance.[14] In his opinion the growths originated from the columnar epithelium lining the upper part of the nasopharynx. The presence of both lymphoid and epithelial elements was not observed in metastases other than lymph nodes.

Teoh in 1957 studied the histopathological features of nasopharyngeal carcinoma in a large autopsy series and noted that within the tumour there was much morphological variation.[15] In one case described (Case 1) he stated that in some areas of the tumour there was cornifying squamous cell carcinoma whilst in others there was undifferentiated carcinoma containing both small and large cells in ill defined clumps mixed with lymphocytes and plasma cells. The metastasis from this primary growth showed similar features to the primary growth but without areas of keratinization. In the liver metastases there were nests and trabeculae of closely packed cells without clear cell boundaries. His histological studies of the primary growths gave clear evidence that all the tumours were carcinomas and in 4 of the 31 cases showed frank epidermoid features. In one case glycogen containing clear cells were seen. Tumours similar to the lymphoepithelioma of Schmincke and Regaud were identified and a transition to the transitional cell carcinoma of Quick and Cutler was documented in one case—indicating that pathologists were probably dealing with one type of tumour and not three. In cases where there were metastases to nonlymphoid tissue there were tumour cells only and not lymphocytes,

even though lymphocytes were present in the primary growth and in cervical lymph node metastases. Teoh's observation concluded that lymphocytes were not true components of the tumour and that their association with tumour cells in sites containing lymphoid tissue was incidental.

In 1962 Shu Yeh from Taiwan published a large (1,000 cases) series of biopsies from patients with nasopharyngeal carcinoma and divided the tumours into three groups; carcinomas, sarcomas and carcinosarcomas.[16] The carcinomas were subdivided into 7 categories depending on histological appearances. Yeh believed that the entity lymphoepithelioma did not really exist and that the 2 criteria which had been used in the past; radiosensitivity and the intimate mixture of lymphocytes were not substantiated. Even though he found that his series of lymphoepitheliomas had a better 5 year survival than the other tumours, because he was very rigid in his inclusion of what should be called a lymphoepithelioma he considered that the low numbers made an accurate assessment of prognosis difficult. He considered the lymphoepithelioma to be nothing but a transitional cell carcinoma originating from deeper crypts of the epithelium, growing down to deeper layers on one hand and infiltrating the superficial lymphoid tissues on the other. As he also found the admixture of lymphocytes and tumour cells to be present in adenocarcinomas, in his opinion the separation of lymphoepithelioma into a separate category was not justified.

Liang, Zhong and others from Canton[17] classified NPC's into three groups, undifferentiated, poorly differentiated and well differentiated which they believed also reflected their biological behaviour; the undifferentiated carcinomas showing cranial base invasion and distant metastases, poorly differentiated either spreading to the cranial base or to lymph nodes and squamous cell carcinoma which usually neither invaded the cranial base nor metastasized distally.

An electron microscopic study of 3 different types of undifferentiated carcinoma was done by Svoboda[18] and this showed cytoplasmic keratin fibrils, features indicative of squamous differentiation. Gazzolo[19] looked at normal and abnormal nasopharyngeal epithelium and identified keratin fibrils, tonofilaments and desmosomes in the tumour cells. When the tumour was undifferentiated there were fewer keratin fibrils, but the presence of desmosomes confirmed the epithelial nature of these tumours. Various types of nuclear bodies were also noted but no Herpes type viral particles were identified. Prasad[20] examined nasopharyngeal carcinoma specimens using transmission electron microscope and confirmed the presence of desmosomes in all cases, tonofilaments in all but 3 cases, rare secretory granules and cytoplasmic inclusion bodies. His conclusion was that the normal pseudostratified columnar epithelium had undergone metaplastic change to the squamous type before undergoing malignant transformation. Michaels and Hyams[21] examined 6 cases and the electron microscopic features correlated well with the histopathological findings. Ultrastructurally, the vesicular nuclei seen histologically revealed small deposits of chromatin beneath the nuclear membrane but little or no chromatin elsewhere. Nucleoli were prominent. Cell processes were not distinct and the cells did not appear to show a definite cell border but appeared to merge into one another. As with previous studies desmosomes were present but tonofilaments uncommon. Both of these investigators, as well as Prasad, attributed the nuclear changes to an increased metabolic activity of the cells. The conclusion was that the undifferentiated carcinoma was a form of squamous cell carcinoma showing minimal differentiation and evidence of high metabolic activity.

THE FIRST INTERNATIONAL CLASSIFICATION

In 1978 the World Health Organisation proposed a histopathological classification which divided nasopharyngeal carcinomas into three categories; squamous cell carcinoma, nonkeratinizing carcinoma and undifferentiated carcinoma.[22] WHO correctly emphasized, by definition, that NPC was a malignant neoplasm that has its origin from the epithelial layer of the nasopharynx, an epithelial layer that might be stratified squamous type, ciliated respiratory type, or some gradation between these two extremes.

Squamous Cell Carcinoma

This was a recognized morphologic category of nasopharyngeal carcinoma and has also been labelled WHO Category I. When this entity was proposed in 1978, in geographic areas of low incidence for nasopharyngeal carcinoma, SCC accounted for approximately 25 percent of all nasopharyngeal carcinomas in Caucasian patients but in Orientals living in USA the frequency was 10% or lower. Patients with squamous cell carcinoma of the nasopharynx, in these low incidence regions tended to have a serological antibody "profile" to Epstein-Barr virus that was similar to normal "controls", though this was not absolute.

Nonkeratinizing Carcinoma

Nonkeratinizing carcinoma of the nasopharynx (WHO Category II), was microscopically identified as a tumour which is neither anaplastic or undifferentiated nor keratinizing. By description, this tumour produced neoplastic epithelium that was "transitional" in type. It often resembled transitional cell carcinoma of the urinary bladder. Cytologically, the cells were of moderate size, variable between polygonal and spindled and variable in differentiation. Nonkeratinizing carcinomas that were poorly differentiated often had the appearance of the tumour described by Regaud. Many pathologists and authors have suggested that nonkeratinizing carcinoma is but a variant of undifferentiated carcinoma, recognizing that some nonkeratinizing carcinomas are cytologically bland (and not undifferentiated). Serologic studies of EBV antibodies show a similar elevated profile of this tumour with undifferentiated carcinoma suggesting that nonkeratinizing carcinoma should be considered separate from the squamous cell carcinoma. In low incidence North American geographic regions, nonkeratinizing carcinoma accounts for 12 percent of all nasopharyngeal carcinomas.

Undifferentiated Carcinoma

Undifferentiated nasopharyngeal carcinoma (WHO Category III) is the most common type of nasopharyngeal carcinoma, accounting for approximately 63 percent of all nasopharyngeal carcinomas in low incidence areas and accounting for up to 98% percent in high incidence areas. It is recognized as a carcinoma that features cells with distinct cytological characteristics; specifically cells with single prominent nucleoli, indistinct cytoplasm and a lack of discernable cytoplasmic outline. The result is a tumour which appears to grow as a syncytium in contrast to squamous cell carcinoma. Many undifferentiated carcinomas incite a reaction of T-lymphocytes. As described above, this lymphocytic response has led to the term lymphoepithelioma, a term which is descriptively inaccurate as the lymphocytes are

not neoplastic. If "lymphoepithelioma" is used in a diagnostic manner, it should be used parenthetically, since these are but variants of undifferentiated nasopharyngeal carcinoma. The term is deeply engrained in pathological literature, however and it is unlikely to be abandoned in the near future. Of interest, undifferentiated nasopharyngeal carcinomas with a lymphoepithelioma pattern are somewhat unique to certain anatomic regions, particularly the region of Waldeyers's ring and the thymus. However, other organ systems (lung, skin, stomach, uterine cervix, breast and bladder) have produced undifferentiated carcinomas with a lymphoid stroma that are microscopically similar to lymphoepithelioma of the nasopharynx. Polymerase chain reaction and in situ hybridization of undifferentiated carcinomas from some of these some of these body sites have been shown to harbour the EB viral genome within the malignant epithelial cells.

At the same time as the 1978 WHO classification was being formulated a group of European pathologists met to discuss the classification of NPC. They agreed that the concept of lymphoepithelioma should be preserved as it described an entity different from squamous cell carcinoma, a reliable and reproducible definition of lymphoepithelioma could be given but that the term lymphoepithelioma was misleading and should be replaced by the term undifferentiated carcinoma of nasopharyngeal type (UCNT). This term would have both a solid cell type (Regaud), isolated cell type (Schmincke) and a spindle cell type. The interobserver agreement on this classification was between 80% and 90%. The other category of squamous cell carcinoma was divided into the traditional well differentiated, moderately differentiated and poorly differentiated carcinoma.[23]

The simplified classification of NPC into squamous cell carcinoma and UCNT showed significant correlation with the EBV serology. The patients with squamous cell carcinoma had low levels of EBV serology while patients with UCNT had elevated titres.[24] Though this finding may have stood ground in Caucasian patients the same cannot be said for Asian patients in whom the EBV genome or its products have been detected in squamous cell carcinoma, though in lower numbers. Thus it appears that in Caucasian patients UCNT and SCC may be two distinct disease processes but in Asian patients, the 2 morphological patterns can be seen within the same tumour specimen. The French classification believed that the WHO Type II (nonkeratinizing carcinoma) was not a genuine entity according to EBV serology and a two tier classification of NPC into differentiated and undifferentiated carcinoma appeared to be the most favourable if a classification based on clinical relevance and serology was to be used.[25]

THE 1991 WHO MODIFICATION

The 1991 modification of the WHO Classification[26] attempted to include some of the findings by European pathologists.[24] The main change was that undifferentiated carcinoma was placed as a subset of nonkeratinizing carcinoma and that the former nonkeratinizing carcinoma was called nonkeratinizing differentiated carcinoma. It is of interest that one of the criteria used for classification in this group was similar to that proposed in the early 1900s—the sensitivity to radiotherapy and that what was called epithelioma in 1903 was now called squamous cell carcinoma (keratinizing squamous cell carcinoma). Another feature of the revised classification was the opinion that the nonkeratinizing tumours have a stronger relationship with the Epstein-Barr virus, a feature which has geographical variation. The 1991 modified scheme still placed what may be considered an undue emphasis on the term lymphoepithelioma. Pathologists

should be aware that this histological subtype is only seen in less than 10% of cases in an endemic area and that the presence of a small number of lymphocytes does not imply that the tumour is a lymphoepithelioma.

THE 2005 WHO CLASSIFICATION

This update to the 1991 WHO classification still maintains the separation between nonkeratinizing carcinoma and keratinizing squamous cell carcinoma but includes as a separate entity a rare tumour called basaloid squamous cell carcinoma. The alternative labelling of the tumours into Types I, II and III has not been emphasized. Within this simplified classification there are a number of statements that bear further investigation, of which the most important relates to the significance of subclassification, as the 2005 classification states that within the nonkeratinizing carcinoma "… subclassification into the undifferentiated and differentiated types is optional, since their distinction is of no clinical or prognostic significance and different areas of the same tumour or different biopsies taken at different time intervals from the same patient may exhibit features of one or the other subtype …".[1] As the main reason for classification is to determine treatment options and prognosis, this statement implies that prognosis of nonkeratinizing NPCs is not determined by whether the tumour has a lymphoid stroma or is transitional in appearance (Fig. 1).

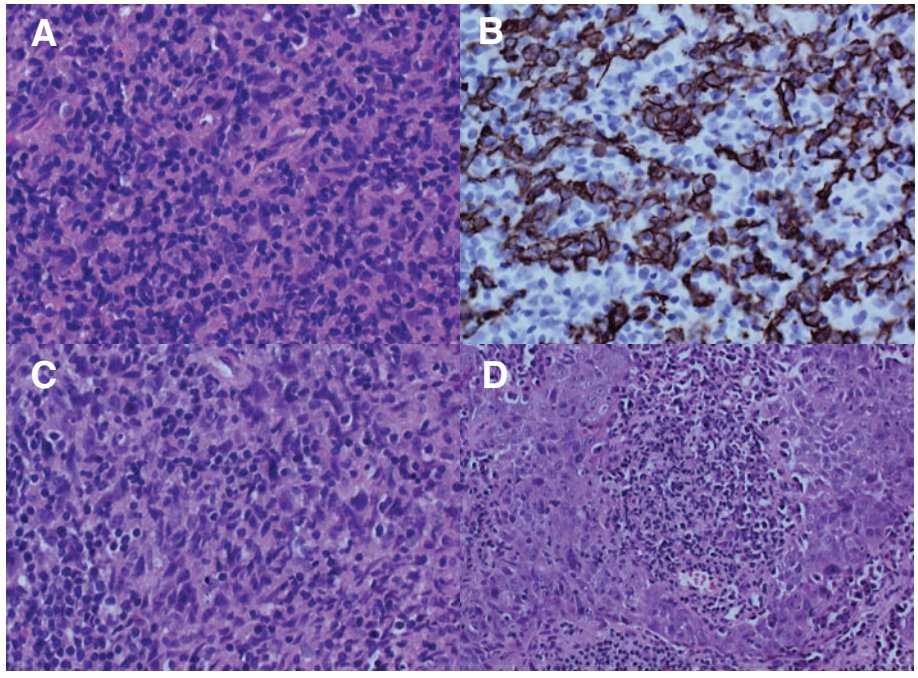

Figure 1. The many faces of nonkeratinizing carcinoma. The tumour may be composed of small isolated tumour cells set among a lymphoid stroma (A). In this case immunohistochemistry for the epithelial marker AE1/AE3 delineates the tumour cells (B). The tumour cells may appear slightly more cohesive (C) and then form nests (D).

Given that there has been considerable confusion since the 1900s over the classification of NPC it is not surprising that the data and results of response to treatment have led to uncertainty regarding whether there is a relationship between the histological subtype of NPC and prognosis. As there have been many classification schemes it is difficult to compare studies as the criteria for sub-classifying a tumour vary from study to study.

The 2005 classification correctly states that "… the density of lymphocytes and plasma cells is highly variable. At one extreme, there are no or few lymphocytes within the tumour islands … and … at the other extreme abundant lymphocytes and plasma cells infiltrate the tumour islands, breaking them up into tiny clusters or single cells and obscuring the epithelial nature of the tumour …".[27] Thus from a purely morphological point of view the justification of Regaud versus Schminke does not appear warranted, but is there a relation between lymphoid infiltrate and prognosis? In 1979 Shanmugaratnam et al stated that the 3 year survival rate for tumours with a marked lymphocytic infiltrate (45.8%) was significantly better than those with a moderate lymphocytic infiltrate (32%) and those with no lymphocytic infiltrate (20.7%).[28] However, as this study was done before CT scanning was routinely performed for accurate staging the real significance of these findings is not clear.

In 1986 a report from Japan by Nomori et al focused not on the lymphocytes but the histiocytic infiltrate in NPC. Using the antibodies S100 and lysozyme, the survival of patients with NPC was related to the density of T-zone histiocytes (Langerhan's cells and their precursors); the more S100 positive cells there were within the tumour, the better the prognosis. The intensity of lysozyme positive cells however was not considered to be related to prognosis.[29] This finding, however, was quickly refuted by a group of European pathologists[30] who found no statistical correlation between the number of S100 positive cells and patient survival but upheld by another European group led by Giannini.[31] These authors first looked at lymphocytic infiltration; finding it to be of no prognostic significance but cases in which a moderate to marked density of dendritic and monocyte/macrophage cells were present showed a prolonged survival.

Studies from the Guangzhou region in Southern China[27] have looked in detail at the stromal response or reaction to the tumour and the findings are in closer agreement with Nomori et al and Giannini et al. This study has shown prognostic significance of the undifferentiated carcinoma when the infiltrate amongst the tumour cells is considered. When the stroma contained abundant lymphocytes, the 5 year survival rate was 59.5%, which was higher than that of the moderate lymphocytic type (51.5%, or when scanty lymphocytes were present (40.1%). This study in effect states that the Schmincke pattern with an abundant lymphocytic infiltrate within the tumour should do better than the Regaud type and not only the lymphocytic infiltration but also the infiltration of macrophages (using lysozyme and S100 protein immunohistochemistry) appears to confer a better prognosis to the patient. The plasma cell population is also not insignificant as the VCA-IgA titre rises with the intensity of the plasma cell infiltrate. Tumours with abundant plasma cells have a geometric mean titre (GMT) of 1:66, those with a moderate plasma cell infiltrate a GMT of 1:30 and a scanty plasma cell infiltrate a GMT of 1:13. When the plasma cells are mature the titre tends to be higher than when the plasma cells are immature.[32]

In conclusion, with regard to the nonkeratinizing tumours there appears to be an impasse over subclassification. On one hand, the "lumpers" will merge the nonkeratinizing differentiated and nonkeratinizing undifferentiated into one entity, arguing that both patterns can be seen within the tumour and the subclassification has no relation to prognosis (Fig. 2). The "splitters" will argue that the lymphoid stroma does appear to be related to prognosis. If the 2005 classification is adhered to then this distinction will be

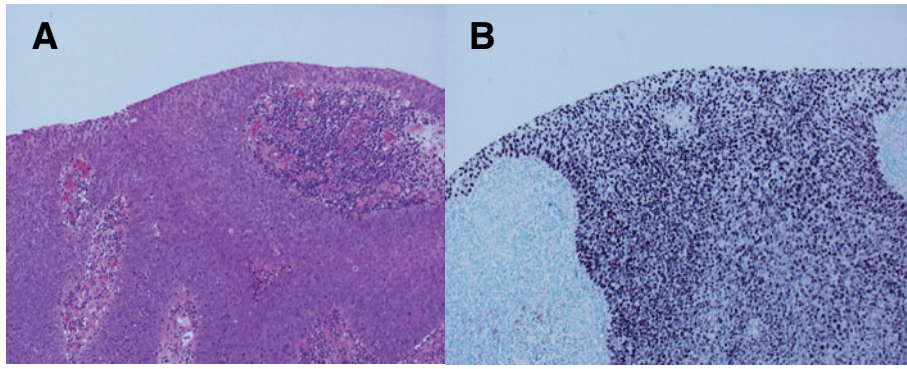

Figure 2. Nonkeratinizing differentiated carcinoma. This tumour characteristically has well delineated nests of tumour cells, often with surface spread. The tumour cells at the periphery of the nests appear palisaded (A). In-situ hybridization for EBER demonstrates that all the tumour cells contain positive signal in the nucleus (B).

lost and future prospective studies will have difficulty in determining prognosis related to histology. In this respect, a large multicentre study from Hong Kong found that T staging was more predictive of tumour relapse and histopathology was not reported to be associated with this relapse, thus supporting the concept of the "lumpers" over the "splitters".[33] Selek and others also found that differentiating between nonkeratinizing undifferentiated and differentiated carcinomas (referred to as WHO II and III) did not appear to have prognostic significance.[34]

KERATINIZING SQUAMOUS CELL CARCINOMA

The 2005 WHO update still separates keratinizing SCC from undifferentiated carcinoma and states that "… the degree of differentiation can be further graded as: well differentiated (most common), moderately differentiated and poorly differentiated…The surface epithelium is frequently involved, apparently representing carcinoma in-situ …".[1] The separation of SCC from undifferentiated carcinoma has been on prognosis, but as with undifferentiated carcinoma this is still controversial (Fig. 3). The work of Liang[17] showed that SCC (WHO I, 1978) was mainly locally invasive, without lymph node metastases but appeared to have a poorer prognosis that the other types of NPC. Similar findings were reported by Meyer and Wang,[35] showing that nonkeratinizing carcinoma has the best prognosis and SCC the least favourable. These results are a reflection of the sensitivity of the carcinoma to radiation therapy which remains the primary form of treatment.

From a practical diagnostic point of view when the tumour has well formed squamous pearls and a surface in-situ dysplastic component the diagnosis will not be contentious, but what happens when the tumour is undifferentiated with small foci of apparent squamous differentiation? (Fig. 4) Should it be called a poorly differentiated squamous cell carcinoma or an undifferentiated carcinoma with focal squamous differentiation? The 2005 classification states that within nonkeratinizing carcinomas there "… can be small foci of primitive squamous differentiation, where small groups of tumour cells exhibit greater amount of lightly eosinophilic cytoplasm and slightly

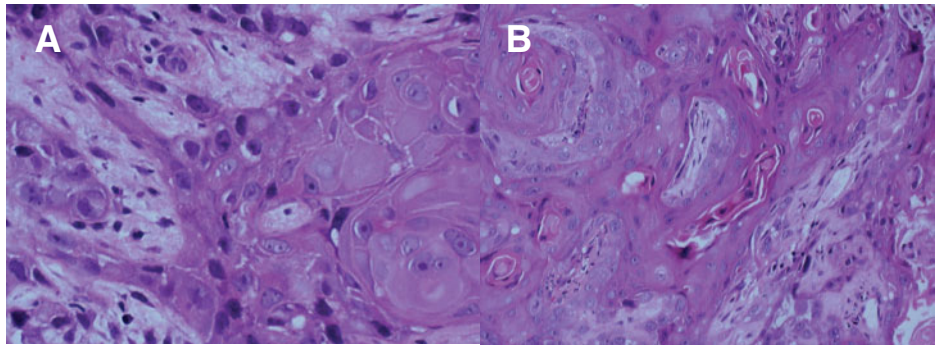

Figure 3. Keratinizing squamous cell carcinoma. The tumour cells show increased eosinophilic cytoplasm with a glassy appearance in keeping with keratin production. Often the stroma is more fibrotic and less lymphoid (A). Orangeophilic keratin is seen in this focus (B).

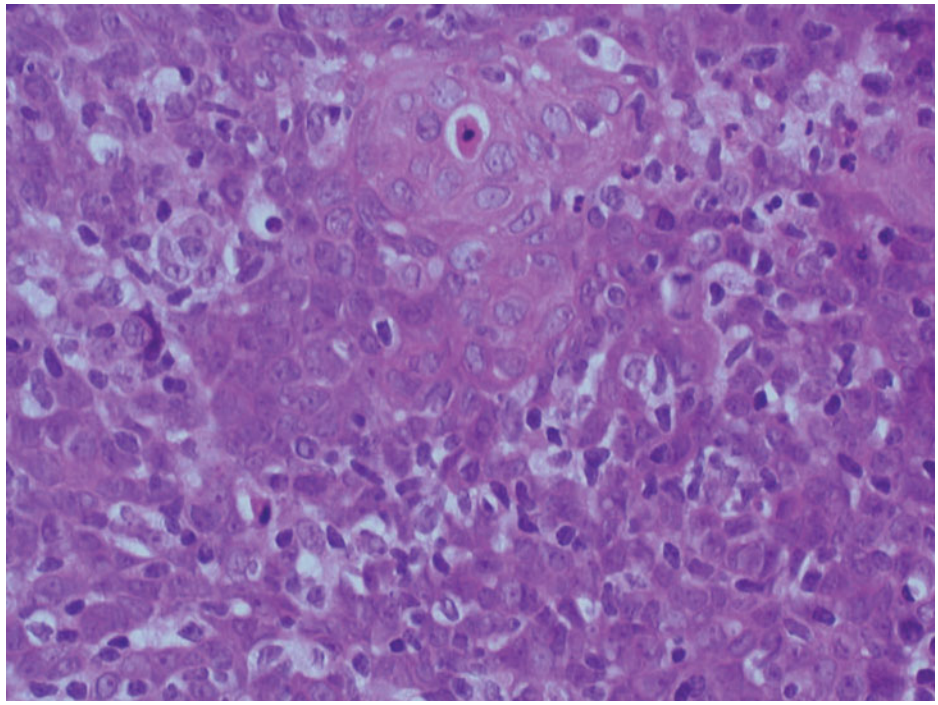

Figure 4. The contentious tumour. In this biopsy most of the tumour is nonkeratinizing but small foci of cytoplasmic eosinophilia and pearl like arrangement are seen. Whether this tumour should be called poorly differentiated squamous cell carcinoma or undifferentiated carcinoma with focal squamous differentiation is subjective.

more distinct cell borders …".[1] To the authors, this is an unresolved grey zone and we tend to compromise by stating that this is an "undifferentiated to poorly differentiated squamous cell carcinoma" realizing that this is perhaps not an ideal situation but

with the understanding that this is not going to affect patient management as there is no difference in treatment for the squamous cell carcinomas compared with the undifferentiated carcinomas. In this respect in situ hybridization studies for EBER will not be rewarding as the squamous cell carcinomas in Hong Kong and other regions with a high incidence of NPC do contain EBV and there is positive staining for EBER in these contentious tumours.[36] We envisage that it is possible that future studies may concentrate on whether the presence or absence of EBV in these tumours may be more of a prognostic guide rather than pure histopathology.

ANCILLARY TECHNIQUES

One of the benefits of the 2005 WHO classification is the inclusion of ancillary immunohistochemical and in-situ hybridization (ISH) techniques for the diagnosis of NPC and the separation from non-epithelial tumours—primarily lymphomas. The cytokeratins are beneficial for separating NPC from lymphoid lesions and commercially available EBER ISH probes can distinguish reactive epithelial lesions from neoplastic ones. The WHO 2005 monograph states that p63, a basal cell marker is useful[37] but the authors have also found that Bcl-2 is another marker that is useful for separating reactive epithelial lesions from neoplastic ones.

CONCLUSION

The 2005 WHO Head and Neck monograph present a crucial decision point for the classification of NPC. Just under 100 years have passed since the first classification of NPC was attempted. Since that time, several classification schemes have been proposed, all failing to find acceptance and utilization by all pathologists around the world. If the current recommended classification by WHO is accepted then the previous attempts at sub-classification will be relegated to historical curiosity as the contention is that they have no diagnostic importance. It is probably not possible to develop a classification scheme, based solely on light microscopy, which totally eliminates subjectivity and inter-observer discrepancies. For the individual patient it is the authors' opinion that the clinician is mainly concerned with separation of NPC from lymphoma and subclassification does not affect initial patient management. Whether the different subtypes (squamous versus undifferentiated) do have any prognostic importance will most likely only be answered by large scale, multi-centre meta-analyses where retrospective analysis of biopsies can be performed.

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CHAPTER 3

DESCRIPTIVE, ENVIRONMENTAL AND GENETIC EPIDEMIOLOGY OF NASOPHARYNGEAL CARCINOMA

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Abstract: Nasopharyngeal carcinoma (NPC) is a rare tumor in most countries but is more prevalent in Southeast Asia, North Africa and Artic regions. Multiple factors participate in the etiology of NPC including Epstein-Barr virus (EBV) activation, genetic susceptibility and exposure to environmental carcinogens. Specifically, risk factors consistently associated with NPC in endemic areas include early childhood salted fish consumption, preserved foods consumption, lack of fresh fruit or leafy vegetables intake, prior chronic respiratory tract conditions, and exposure to cooking fumes. EBV may act as a tumor promoting agent rather than an initiator in the progress of NPC carcinogenesis. Genetic susceptibility to NPC is largely mediated by the human leukocyte antigen (HLA) class I genes region, although it is not clear whether HLA is causative.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) exhibits a distinct geographic incidence patterns across the world. It is a multifactorial disease whose etiology involves the complex interplay among Epstein-Barr virus (EBV), genetic susceptibility and environmental factors. Association between low socioeconomic status (SES) and NPC has been observed throughout all endemic areas in the world and is well established,[1] though without a real understanding of the underlying causes. This chapter reviews the epidemiological studies on NPC, with a focus on their implications for etiology.

Nasopharyngeal Carcinoma: Keys for Translational Medicine and Biology,
edited by Pierre Busson.

DESCRIPTIVE EPIDEMIOLOGY

Geographic Distribution

NPC is rare in most parts of the world, but much more common in Southeast Asia, Maghrebian countries (Algeria, Morocco and Tunisia) and the arctic and sub-arctic region of North America and Greenland. The major endemic ethnic groups are southern Chinese, Amazigh- and Arabic-speaking North Africans and Inuit, respectively. One of the highest incidence rates of NPC during 1998-2002, demonstrated as age-standardized rate (ASR) of 26.9 per 10^5 person-years for males and 10.1 for females, came from Zhongshan city of Guangdong province, southern China,[2] where the majority of the population is Cantonese. Comparable ASRs were observed in nearby cities including Hong Kong and Guangzhou. The circumpolar Inuit in Alaska, Canada and Greenland also show high incidence of NPC, where the ASR was 12.1 for males and 7.3 for females during the period 1989-2003.[3] Populations from some North African countries demonstrate intermediate risks of NPC: ASRs of 5.4 and 4.6 per 10^5 person-years during 1998-2002 were reported for males in Algeria and Tunisia respectively, while ASRs for females were 1.7 and 1.9.[2] In contrast, the incidence for most other countries is less than $0.5/10^5$/year (Fig. 1). In most populations, the male to female incidence ratio is about 2-3:1.[2]

In Southeast Asia, the population structure is diverse, and the NPC risk varies. In Guangdong province of southern China, the majority of the population is Cantonese (guǎng-fǔ), followed by Hakka (kè-jiā) and Teochew (cháo-zhōu) people, each of which has its own dialect. Chinese living in other Southeast Asian countries are normally a mixture of these people together with Hokkien (fú-jiàn), another ethnic Chinese originated from Fujian province. Cantonese has an NPC risk twice those for the other groups, in China and in other countries.[5] Besides Chinese, Thais, Vietnamese, Malays and Filipinos also show an intermediate to high risk of NPC, where the ASR ranges from 2.5 to 15 for males. Noteworthy, while the Sarawak province of Malaysia recorded ASRs of 15 for male and 6.5 for female during 1998-2002,[2] one of its native populations, the Bidayuh, has a much higher risk (ASR 31.5 for males and 11.8 for females) than the other ethnic groups living in Sarawak.[6] Similarly, in northeast India, a high ASR of 19.4 was recorded in the Kohima district in Nagaland State, where the major population is the Nagas people. Even higher ASR of 21.7 was recorded in the Serchhip district, where the Mizo people reside. ASRs in nearby districts in northeast India range from 1 to 20.[4]

There exist distinctive age-incidence curves for NPC in different populations (Fig. 2). In Asian high-risk populations, incidence rises in adolescence, peaks at 45-55 years, and declines subsequently; while in low-risk populations, irrespective of geographic location and sex, the age-incidence curves are bimodal, where the first peak normally appears at age 15-24 years, and the second at 65-79 years, followed by respective declines.[7] In the intermediate-risk Maghrebian populations, NPC is also characterized by a bimodal age distribution, with one peak occurring in the teens and the other at age 45-60 years.[8] In all populations featuring bimodality, the incidence of NPC at the second peak is higher than the first one. The decline of NPC risk after a certain age is in contrast to some other malignant tumors such as colon cancer, whose risks increase monotonically with age. It could be interpreted as a frailty phenomenon, whereby only a small fraction of the population is in high risk of the disease at a given age, such that the overall population risk must decline after the exhaustion of the susceptible individuals.[7] The bimodality in low- to intermediate-risk populations may suggest a heterogeneous etiology within the

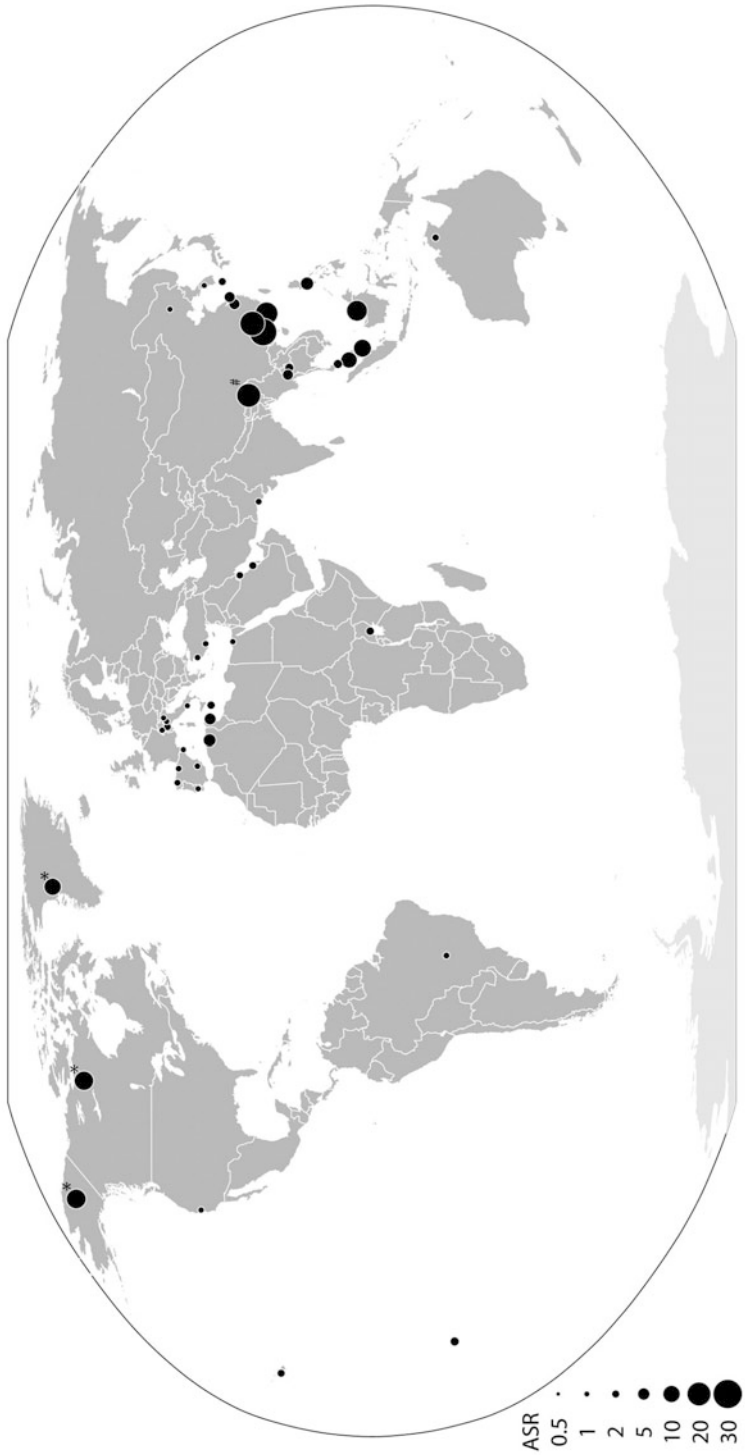

Figure 1. Incidence of NPC in males in the world. ASR: age-standardized (world) incidence rate. Only ASRs greater than 1.0 are shown. Unless otherwise stated, data were taken from the *Cancer Incidence in Five Continents, Volume IX*.[2] *Circumpolar Inuit, data taken from Kelly et al.[3] #Northeast India, data taken from Nandakumar et al.[4]

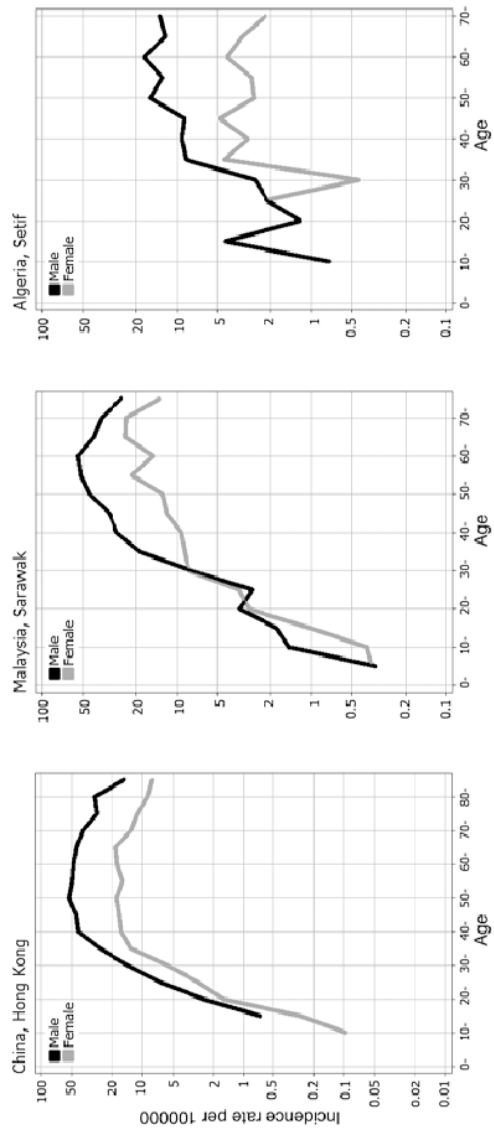

Figure 2. Age-dependent incidence rate of NPC in different populations. The upper part was created by CANCER*Mondial* (http://www-dep.iarc.fr/); the lower part is the combined incidence rates of several low-risk areas including India, Japan, North America, Northwest Europe and Australia, which was adapted with permission from *Cancer Epidemiol Biomarkers Prev* 2008;17(9): 2360 Figure 2A.[6]

same population. However, although it is not immediately evident, bimodality cannot be excluded definitely from high-risk populations; it is hypothesized that it is also a feature of NPC in these populations, and that the first peak is simply masked by the overall high-risk status.[7]

The World Health Organization has classified NPC into three histological types (the 2005 WHO classification)—keratinizing squamous cell carcinoma (KSCC), nonkeratinizing carcinoma and basaloid squamous cell carcinoma—and subdivided nonkeratinizing carcinoma into differentiated nonkeratinizing carcinoma and undifferentiated carcinoma. However, the boundaries between categories are not always clear and some investigators view them as variants of a fairly homogeneous group of tumors.[9] Notwithstanding this problem, nonkeratinizing carcinoma is the major histological type in Southeast Asia,[10] northern Africa[11] and the arctic,[12] while keratinizing squamous cell carcinomas is more common in low-risk populations such as US whites.[13] Nonkeratinizing carcinoma is invariably associated with EBV infection. In fact, in the 1991 WHO classification, the reason to combine nonkeratinizing carcinoma and undifferentiated carcinoma (Type 2 and 3 in the 1978 WHO classification) into one category is that they exhibit similar epidemiologic and biologic characteristics, including the EBV relationships.[14] On the contrary, the association between KSCC and EBV varies between populations, and hence it is viewed as a pathologically heterogeneous group of tumors.[15] Basaloid squamous cell carcinoma is relatively uncommon in both endemic and non-endemic areas.[9]

Time Trends

Recently, there is a documented decreasing trend of NPC risk in several endemic areas of Southeast Asia,[16] Greenland[12] (although not significant), and among Chinese living in Los Angeles and San Francisco.[17] One explanation for such decreases may be the changes in childhood environmental exposures due to the economic development in Asia, since that the observed declines commonly started 30 years after the onset of rapid economic growth in Hong Kong, Taiwan and Singapore that occurred in the 1940s, 1950s and 1960s, respectively.[16] On the other hand, the incidence of NPC has remained stable or slightly increased during the period 1983-2002 in Sihui and Cangwu counties of southern China,[18] whose economic growth started after 1978 and for which the incidence of NPC is expected to decline later.

Another source of the decreasing trend may be tobacco control. The decline in NPC incidence among Chinese in the USA is limited to keratinizing carcinoma, a histological subtype of NPC that is unambiguously associated with tobacco in different populations. The same was observed for the decline in Hong Kong after 1988, which parallels the reduced prevalence of cigarette smoking since the 1970s, allowing for a 10-years lag time.[19] However, due to its low consumption rate, tobacco cannot fully explain the decreasing NPC risk in this region.[20]

Influence of Migration

In low-risk areas, immigrants from high-risk regions remain susceptible to NPC, as has been observed for Chinese in the USA, UK, Canada and Australia,[16] Inuit in Denmark,[21] and North Africans in several European countries.[1] Their NPC incidence rates are much higher than the native populations, but normally slightly lower than their

counterparts living in the original country. Interestingly, successive generations of these immigrants show a declining trend in NPC risk, which could be explained by the gradual abandonment of their original lifestyles, dilution of genetic causal variants by admixture with other low-risk populations, or even the potential diminishment of a specific EBV strain over generations. Thus, to generate clues about NPC etiology, it may not be very helpful to compare the incidence rate of all immigrants from high-risk areas with that of their counterparts.

A more relevant approach is to investigate only the first-generation immigrants, who are distinguished from their nonmigrant counterparts solely by environmental exposures acting late in life, but not genetic susceptibility or early-age exposures. Therefore, if the disease risk significantly reduces after they relocate to a low-risk country, it will point to the importance of adulthood exposures. However, no obviously reduced risk for NPC was found for China-born immigrants living in Southeast Asian countries,[5] or Inuit living in Denmark for decades,[21] suggesting the importance of genetic factors or childhood environmental exposures. It should be noted that, in high-risk regions such as Southeast Asia and Greenland, EBV infection takes place early in life; therefore, the childhood environmental exposures that maintain the high-risk status of these first-generation immigrants could be lifestyles and/or EBV infection. Nonetheless, EBV alone is not sufficient to cause NPC, since both Malays and Chinese living in Singapore are affected with EBV at approximately the same age, but Chinese have a much higher risk than Malays, hinting at the involvement of genetic susceptibility and other childhood exposures.[22]

Another approach is to study individuals of a low-risk population born in a high-risk area, such that early childhood exposures are the only differences when these individuals were compared with their counterparts who have not been in a high-risk area. A study showed that White male descendents born in the Philippine islands or China have an increased death rate of NPC. Similarly, for individuals of French origin living in the South of France, being born in North Africa (Algeria, Morocco and Tunisia) can increase NPC risk almost six fold. In addition, for English and Welsh males, being born in the Indian subcontinent is associated with a significantly higher risk of NPC.[1] These all imply the involvement of early childhood exposures in disease etiology.

ENVIRONMENTAL FACTORS (NONVIRAL AND VIRAL)

Dietary Risk Factors

Given its distinct geographic distribution, one may speculate about the common features among the endemic populations. Anthropology studies observed that in China, Greenland and Tunisia, NPC is associated with a low socioeconomic status (SES), which may have a link with overcrowded habitation, lack of ventilation, poor hygiene, and a lifestyle characterized by monotonous diet and consumption of preserved foods, which are among the least expensive foods available in these populations.[1]

Chinese-style salted fish, preserved by salting and then sun-drying, is one of the cheapest preserved foods in southern China, and has been widely reported to be associated with an increased risk of NPC among Cantonese living in Southeast Asia, as well as among other populations including Thai.[1] Noteworthy, in northern Thailand, there are three groups of salted fish according to the type of fish: two fresh-water fish and one saltwater fish. The latter is prepared in the same way as Chinese-style salted fish and is

the only one that is significantly associated with an elevated NPC risk.[23] In some studies, the association remained significant after adjusting for socioeconomic status and other risk factors, and a dose-response relationship between intake frequency and NPC risk was observed.[24] Several studies found that only the consumption of salted fish during childhood and weaning is significantly associated with NPC, while consumption during adulthood is not.[1] Besides the age of consumption, the manner of cooking and the type of fish may also be important; sea salted fish carried a higher risk than fresh-water fish, as well as steamed salted fish than fried, grilled or boiled salted fish.[1] Summarizing the above evidence, Chinese-style salted fish was then classified as carcinogenic to humans by the International Agency for Research on Cancer (IARC) of the World Health Organization.[24]

Regarding other types of salted fish in the rest of the world, among the Alaskan native population, there is a suggestive higher frequency of salted fish consumption during childhood in NPC patients than in controls.[25] In Greenland, Eskimos often consume wind dried and slightly fermented fish and seal meat since weaning,[1] but its association with NPC has not been documented. In northern Africa, the frequency of salted fish consumption is low. In Japan, salted fish is frequently consumed; however, Japanese have a low incidence rate of NPC. This observation may be explained by the type of fish, method of preparation, method of cooking, age at consumption, and the higher amount of fresh fruits or vegetables intake among Japanese.[1]

Besides salted fish, other preserved foods and condiments are related to elevated NPC risks in many regions. These include fermented fish sauce, salted shrimp paste, moldy bean curd, and preserved plums in childhood diet in Guangzhou, salted duck eggs, salted mustard green, chung-choi (brine fermented radish root), dried fish, and fermented soy bean paste during weaning or childhood in Guangxi Yulin, and salted eggs during childhood among Malaysia Chinese.[1] Associations of all these foods remained significant after adjusting for salted fish consumption. It seems that, in addition to salted seafood, intake of preserved vegetables during early years is the common characteristic of diet among Chinese NPC patients, albeit insufficient controlling for potential confounding factors is one of the blemishes in some of the above studies. Recently, after careful assessment of study qualities including proper controlling for the potential confounding by EBV, salted fish and other factors, a meta-analysis evaluating adulthood (not childhood) consumption of preserved vegetables yielded a pooled odds ratio of 2.04 [1.43-2.92].[26] Although this provides further evidence for the harmful effect of preserved vegetables, it is still not clear whether childhood or adulthood intake is more important to the etiology of NPC.

Preserved foods have also been found to be risk factors in populations from northern African countries, such as quaddid (dried mutton stored in oil), harissa (very spicy condiment prepared with red pepper, olive oil, garlic, caraway) and toklia (basic stewing preparation, contain red pepper, black pepper, garlic, salt, oil, caraway and coriander) introduced in childhood diet.[27] However, in a larger study conducted in Algeria, Morocco and Tunisia, the association of these food items became nonsignificant in a multivariate analysis. Instead, increased risk associated with rancid butter and rancid sheep fat intake during adulthood remained in the multivariate model.[28]

Similar to other cancer types, various epidemiological studies have consistently found that fresh fruit and leafy vegetables are protective factors against NPC in Chinese,[29-34] North Africans,[28] and some low risk populations,[35] which was then confirmed by a meta-analysis.[26]

The identification of carcinogenic ingredients in the associated foods was one of the study concerns in previous publications. Investigators have analyzed food samples from endemic areas, and found that some volatile N-nitrosamines, namely

N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidine (NPYR) and N-nitrosopiperidine (NPIP), compounds that are classified as probably or possibly carcinogenic to humans by IARC,[36] were detected in toklia, quaddid, Chinese salted fish, brine fermented vegetables and Greenland dried fish.[37-40] In addition, an in-vitro nitrisation study indicated that a quantity of N-nitrosamines could be fermented within the human stomach from their precursors contained in Chinese salted fish and Tunisian spice.[41] Recently, a case-control study conducted in Taiwan found that, the highest quartile intake of nitrosamines from meat, fish and preserved vegetables during weaning can confer a 3.9 fold increased risk of NPC (95% confidence interval = 1.4~10.4), which is much higher than any individual food item alone, including salted fish.[42] All these results support the hypothesis that nitrosamines and their precursors are the responsible ingredients in the associated foods.

In addition to N-nitrosamines, other substances in these preserved foods could also contribute to NPC. It has been found that extracts of Cantonese salted fish, harissa and quaddid from Tunisia are capable of inducing EBV early antigens in Raji cells;[43] however, there is no correlation between level of N-nitrosamines and EBV-inducing activities, before and after nitrisation.[41] Therefore, the EBV inducers are thought to have chemical structures different from N-nitrosamines. Afterward, lignin-containing high molecule complexes were isolated from harissa and were shown to be strong EBV inducers.[44] Nevertheless, exactly what substances in these foods are reactivating EBV in the human body is not yet known.

The association of rancid butter and rancid sheep fat with NPC in northern Africa may suggest another carcinogenic compound, butyric acid, the glyceride form of which makes up 3-4% of butter, and is released into free form by hydrolysis when the butter becomes rancid.[28] Butyric acid is known to be able to activate EBV in the B-lymphoid cells into lyric cycle,[45] and therefore, could be related to NPC.

Tobacco, Alcohol, Cannabis and Fumes Intake

Association between cigarette smoking and increased risk of NPC has been consistently reported in some low incidence areas, such as North America,[46-50] where differentiated NPC are the predominant histological type.[13] However, the associations with smoking have been controversial in high incidence areas, where the majority of NPC tumors are undifferentiated.[51-59] The dissimilar contribution of tobacco to different subtypes of NPC was better demonstrated in two studies where the risk of tobacco is defined by histological type. In a low-risk population of the USA, cigarette smoking was associated with differentiated squamous cell carcinoma with a significant dose-response relationship, and the highest dose smoking (current smoker with a history of > 60 pack-years) has an odds ratio of 6.5; whereas the association with undifferentiated or nonkeratinizing carcinoma was not evident.[47] The latter may be explained by the lack of power due to small sample size. However, in another study conducted in northern Africa, tobacco consumption (cigarette smoking or snuff intake) was significantly associated with differentiated NPC but not with undifferentiated carcinomas, even though the latter is the major histological type of NPC in these populations.[11]

Cannabis is one of the most prevalent illicit drugs associated with cancer risk.[60] It can be consumed in herbal form (dried buds or flowers of cannabis), in resinous form, or in oil form. In herbal form, marijuana can be smoked alone, or together with tobacco. Even when it is smoked alone, marijuana produces many of the carcinogens and cocarcinogens

that can be found in tobacco smoke.[60] In the above-mentioned study in northern Africa, marijuana smoking was associated with a significantly elevated NPC risk independently of cigarette smoking, as demonstrated by a stratified permutation test and by conditional logistic regression.[11] In this study, tobacco and cannabis are associated with differentiated and undifferentiated NPC, separately, suggesting dissimilar carcinogenic mechanisms between them.

In regard to alcohol consumption, most, but not all studies reported no association, exceptions include studies in Malaysia[61] and the United States.[47,49] However, increasing the power by combining the results from these small studies, a meta-analysis found an excess risk in a comparison of the highest to the lowest category of total alcohol intake (OR = 1.33 [1.09-1.62]). When analysis were restricted to studies that controlled for cigarette smoking, a strong confounder of alcohol intake, the association was borderline significant.[62] The data suggested a potential J-shaped dose-response trend, whereby high alcohol intake is associated with an increased NPC risk, while light intake is inversely associated, a finding that is rare for most cancers.[62] It should be noted that, the OR for highest-vs.-lowest alcohol intake may be an overestimation of association due to the methodological limitations of the selected studies, such as the lack of controlling for potential confounding factors, the lack of measurement of alcohol intake, and publication bias.[62] Therefore, the relationship between alcohol and NPC risk warrants further confirmation.

It has been postulated that NPC patients in southern China were more exposed than controls to domestic fumes intake, either by poor ventilation in kitchen (absence of windows and chimney), cooking in the main room, or cooking with wood fire.[30,34,63] Similarly, northern African NPC patients reported poorer ventilation status than controls, indicated by usage of a traditional cooking facility—kanoun, absence of windows and chimney in kitchen, and wood fire cooking. Additionally, childhood exposure conferred higher risk than that during adulthood, a difference that cannot be explained by the aggregate exposure time, because the NPC risk remained significantly elevated among individuals who use kanoun during childhood but not during adulthood.[11]

Epstein-Barr Virus

Epstein-Barr virus is a double-stranded DNA herpes virus that infects more than 90% of all humans and results in life-long virus persistence. Due to different levels of hygiene and crowding that may affect salivary contact, some populations are infected during their childhood years, causing no to mild symptoms,[16] whereas in developed countries infection takes place later and can cause infectious mononucleosis.[64] In most cases, EBV stays in latency in peripheral blood lymphocytes causing no serious consequences; while occasionally, EBV can become active and may contribute to several malignancies.

The oncogenic role of EBV in the genesis of undifferentiated nasopharyngeal carcinoma is well established and is evident by the following observations: (1) Antibodies against EBV are elevated in patients,[65] even years before diagnosis.[66] (2) EBV DNA and RNA are present in almost all tumor cells[67-69] and some, if not all, pre-invasive lesions (carcinoma in-situ or high-grade dysplasia),[70-72] but not normal epithelial cells adjacent to NPC,[72] nor biopsies from nonNPC individuals.[73-75] (3) The monoclonality of the viral genome in NPC tissues suggests that EBV infection takes place before the clonal expansion of malignant cells.[69,70,76,77] All these facts support the concept that EBV infection in epithelial cells is an early event in NPC carcinogenesis, but probably not the first step,

since the stable EBV infection may require an undifferentiated cellular environment,[78,79] and EBV infection is absent in low-grade dysplastic lesions.[80] After infection, EBV can deregulate a series of key proteins involved in apoptosis, cell cycle checkpoints, and metastasis,[81] acting as a tumor-promoting agent rather than an initiating factor in the oncogenic process.[82]

While EBV is consistently detected in undifferentiated carcinomas regardless of geographic origin, its association with keratinizing squamous cell carcinoma is less consistent, particularly in non-endemic areas.[83] Considering that EBV infection is ubiquitous while the EBV-associated NPC has a distinct geographical distribution, it is postulated that a certain EBV strain may exist in higher frequencies in endemic areas and contribute to the prevalence of NPC. In this regard, no definitive answer can be obtained so far due to the limited genomic and geographical regions studied, the small sample sizes used, the variety of specimen types investigated, and the complexity that multiple EBV strains can infect an individual and may have different preferential infection sites or compartments.[84]

Occupational Exposures

Formaldehyde is a chemical compound that is used in pressed-wood products, glue and adhesives, pulp and paper, textile finishing, disinfectants and preservatives, etc. Experimental animal studies showed that high-level inhalation of formaldehyde induces squamous-cell carcinomas of the nasal cavity in rats. In epidemiological studies in humans, most case-control studies reported elevated risks of NPC associated with formaldehyde. In addition, two cohort studies of workers exposed to formaldehyde in the USA and Sweden found significant excess deaths from nasopharyngeal cancers. Although some other cohort studies found no excess risk, the power of those studies is low. A meta-analysis including some of the above studies found a significant meta-relative risk. Therefore, the IARC monograph-working group concluded that there is sufficient evidence in humans and experimental animals for the carcinogenicity of formaldehyde, mainly due to an excess risk of NPC.[85]

Other occupational exposures are less widely replicated or confirmed. These include wood dust—an exposure known to be related to adenocarcinoma of the nasal cavities and paranasal sinuses, cotton dust, industrial heat and combustion products. Excess risks of NPC have been observed for several categories of workers, such as printing or agricultural workers, among others. But the specific substances responsible for the association have not been identified.[16]

Other Exposures

Prior chronic ear, nose, throat and lower respiratory tract conditions are associated with a doubled risk of NPC in many studies in Chinese, as well as those in Kenyan or US men.[16] Although it is well established that chronic inflammation predisposes tissue to various types of cancers,[86] the specific mechanisms for NPC is not known.

People with acquired immunodeficiency syndrome (AIDS) carry a higher risk of virus-attributable cancers, such as Kaposi sarcoma, lymphomas and anogenital cancers. Follow-up of a large cohort of AIDS patients in the US has discovered that individuals with AIDS have approximately two-fold risk for salivary gland cancer and

nasopharyngeal cancer, which are both associated with EBV, suggesting an important role for immuno-suppression and viral etiology.[87] Similarly, individuals affected with dermatomyositis (DM) are at a 66-times higher risk of NPC, the highest among all cancer sites for DM patients. Genetic susceptibility may be one of the explanations for this association as both diseases are associated with the major histocompatibility complex (MHC) region. In addition, EBV infection may be involved also in DM, though the underlying reason linking DM with NPC is still obscure.[88]

Other suggested exposures remain unclear and include traditional herbal medicine, betel nut chewing (classified as carcinogenic due to oral cancer by IARC),[89] nickel levels in drinking water or other trace elements.[16]

GENETIC EPIDEMIOLOGY

Familial Clustering of NPC

Familial aggregation of NPC has been widely documented in Chinese: more than 5% of the NPC patients have a positive first-degree family history of NPC in high-risk areas such as Hong Kong (7.2%), Yulin (6.0%) and Guangzhou (5.9%),[56,63] which could be explained by the shared environmental exposures, specific EBV strains and/or higher genetic predisposition among family members, as well as the high prevalence of NPC in this population. While first-degree family members of an NPC patient are at risk of the disease twice of the general population, this risk is further elevated if the patient is of an early onset (<40 years of age), suggesting a potential genetic contribution.[90] Familial aggregation of NPC with another EBV-related tumor, salivary gland carcinoma, has only been documented in the Inuit population.[12]

Chromosomal Aberrations in NPC Tumors

NPC is characterized by chromosomal abnormalities during its development and progression. Some abnormalities, particularly chromosomal gains or losses occur at early stage of NPC, are suggestive of the location of an oncogene or tumor suppressor gene, respectively. To search for these genes, two techniques were widely used to study unbalanced chromosome copy number changes in NPC tissues, comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) analysis.

Most of the CGH studies were performed within the Chinese population, revealing frequent gains at chromosome 1q, 3q, 8q, 12p, 12q and losses at 3p, 9p, 11q, 14q and 16q. These results were further corroborated by LOH analyses, which identified common chromosome losses at 3p, 9p and 14q.[91] Based on the CGH results, evolutionary tree models were constructed to represent the pathogenesis progression of NPC, from which it is predicted that 3p loss and 12p gain are early events in NPC development.[92,93] Interestingly, 3p and 9p losses were also frequently detected in normal nasopharyngeal epithelia from southern Chinese, but not as frequent in low-risk northern Chinese, providing evidence that they are early events in NPC etiology.[80,94] These findings suggest that disruptions of tumor suppressor genes located on 3p and 9p are important during the initiation stage of NPC, which may include *CDKN2A* and *CDKN2B* on 9p21, and *RASSF1A* and *ZMYND10/BLU* on 3p21.3.[95]

The Human Leukocyte Antigen Region

The involvement of EBV in pathogenesis of NPC inspired the association studies of human leukocyte antigen (HLA) class I genes, which encode proteins to identify and present foreign antigens, including EBV peptides. It is hypothesized that individuals bearing HLA alleles more efficient at inducing a cytotoxic immune response against EBV antigens are at reduced risks of NPC, and vice versa. There are consistent reports of association between HLA class I genes and risk of NPC, for both sporadic and familial cases. However, the thought that a specific HLA variant is directly involved in NPC pathogenesis has been challenged, since distinctive HLA serotypes are associated with disease risk in different populations. For example, *A2* increases risk in Chinese, but reduces risk in low- to intermediate-risk populations, and vice versa for *B13*.[96] In addition to these two serotypes, from a meta-analysis, other associated serotypes for Chinese are *A11*, *B18*, *B46* and *B55*, while in intermediate-risk populations they include *A10* and *B14*, with no overlap in associated alleles between the two groups.[96]

These discrepancies may stem from the low resolution in HLA typing and the dissimilar effect associated with different HLA subtypes within a certain serotype. For example, in Chinese, common *A2* alleles include *A*0201*, *A*0203*, *A*0206* and *A*0207*, among which *A*0207* is the only one that confers an increased risk of NPC[97] (although some other studies argue against it).[98,99] Whereas in Whites *A*0201* predominates and may exert a protective effect.[100] This explanation is further bolstered by the observation that *A*0201* may play a major role in immune response to EBV antigens such as LMP1, LMP2 and BARF1 proteins,[100] and *A*0207* is less efficient compared to *A*0201*.[101] However, although this may answer why *A*0201* is protective against NPC and attributes to the inverse association of *A2* in Whites, it still cannot explain the excess risk associated with *A*0207* in Chinese.

Another explanation for the controversial results may be the genetic variability of EBV. It was found that in southern China and Taiwan, NPC biopsies carry in high frequency an EBV strain expressing an HLA *A2*-restricted 'epitope-loss variant' of *LMP1*,[102] which may allow the EBV-infected cells to escape from immune recognition.[103] Furthermore, mutations in HLA-A11 epitopes within the EBV nuclear antigen *EBNA3B* were also reported in Chinese.[104] These are hinting that EBV varies in its HLA epitopes among populations, which may contribute to the genetic heterogeneities of association between HLA and NPC.

Last, but not least, the association of HLA may be interpreted as representing linkage disequilibrium (LD) with another NPC-causal gene(s) located within the MHC region. As the LD pattern varies across populations, the associated HLA allele differs subsequently. Nevertheless, it is not known from the current data whether these HLA alleles are directly involved in NPC pathogenesis, or if they are only markers of another NPC-causal variant. The situation may be even more complicated in that multiple genes within the MHC region may confer an increased or decreased risk for NPC, and the two apparently conflicting hypotheses may both be true.

Chromosomal Regions in Linkage with NPC

Linkage analysis is an approach to disease gene hunting that utilizes families with multiple affected individuals and tests the cosegregation of disease status with chromosome transmissions within a family. There are 4 linkage studies performed

in Chinese hitherto, where the linkage of 3p21,[105] 4p15-q12,[106] 5p13[107] and 6p21[108] were identified, respectively. These diverse findings indicate the potential genetic heterogeneity among Chinese familial NPC cases. Following these results, effects have been made to narrow down the region and to identify the NPC susceptibility genes, but no conclusive answers were provided yet. Noteworthy, the clearly associated HLA region was seldom detected by linkage studies. This may be because linkage analysis is more powerful in searching for rare variants associated with high penetrance, while the association study design is suitable for common variants associated with small to medium risk, and the NPC susceptibility gene within the MHC may belong to the latter scenario.

Genes Associated with NPC Susceptibility

Many genes have been suggested to be associated with NPC, such as those related to carcinogenic metabolism (*CYP2E1*,[109-114] *CYP2A6*,[115] *GSTM1*,[116] *NAT2*),[117] DNA repair (*XRCC1* and *hOGG1*,[118,119] *ERCC1*,[120] *RAD51L1*),[121] cell cycle regulation (*TP53*,[122] *CCND1*),[123,124] immune response (*TLRs*,[125,126] *PLUNC*,[127] interleukins,[128-130] *FAS*[131]), or EBV receptors (*PIGR*,[132] *TCR*[133]). However, most of these associations were reported from small-scale case-control studies, and confirmations are limited. Therefore, they need to be carefully interpreted.

Recent genomic technology developments make possible a large-scale genome-wide association study (GWAS), a powerful approach to search for common variants causing a common disease, without an underlying assumption of the biological relevance of a specific gene or locus. A recent GWAS conducted in 111 Malaysian Chinese NPC patients and 260 controls revealed the increased NPC risk associated with a variant at the *ITGA9* gene locus, located within the commonly deleted 3p21 region.[134] Another GWAS performed in 277 Taiwan Chinese patients and 285 controls suggested multiple loci within the MHC region in association with NPC.[135] It should be noted that, none of these studies involved more than 300 cases in the discovery stage, thus they are underpowered to detect variants conferring small to medium risk for NPC. The third GWAS was performed in southern Chinese, with 1583 cases and 1894 controls at the first stage and 3507 cases and 3063 controls at the second. The joint analysis of the combined samples confirmed the major role of *HLA* in the inheritance of NPC, and found two loci significantly associated with NPC—*TNFRSF19* and *MDS1-EVI1*.[136] These loci were confirmed by TDT (transmission disequilibrium test) tests in an independent validation sample of 279 family trios from Guangdong, which effectively enhance the probability that these findings are true. There may be many other genes with small effect sizes that this study was not able to find; nonetheless, it provides clues to the inheritance of NPC in the general population of southern China. Further genetic and functional study of these genes and their products should yield more insights into the NPC etiology.

CONCLUSION

From all the epidemiological studies performed to date in NPC, only a few risk factors have been consistently associated with NPC, which include early childhood salted fish consumption, preserved foods consumption, lack of fresh fruit or leafy vegetables intake, prior chronic respiratory tract conditions, and cooking fumes exposure. Others are

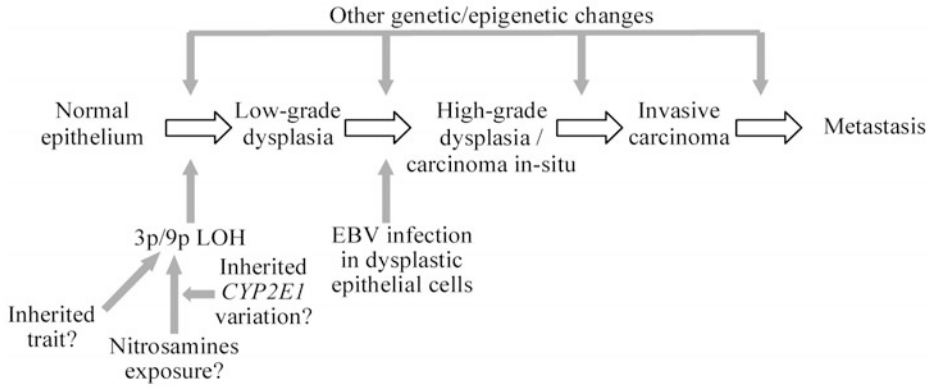

Figure 3. Proposed stepwise pathogenesis of NPC.

controversial or seldom replicated. Besides the failure to control for potential confounders and the lack of power in several individual studies, the controversy may be partly due to the complex etiology of the disease.

NPC has a heterogeneous origin among endemic populations, as indicated by the different associated dietary habits between Chinese and North African populations. Even within a population, heterogeneity also exists, as suggested by the bimodal age-incidence curves, the distinct incidence rate of NPC in different ethnic groups of southern Chinese, and the various epidemiological findings from a geographical region, e.g., the controversial results from Southeast Asia regarding cigarette smoking. Nevertheless, several studies also reported success by separating patients by histological type, age at onset, or even dialect.

The complexity in disease etiology can also stem from the interplay among risk factors. It seems clear that not a single factor can explain all cases of NPC in an endemic area. Thus, the associated risk factors may contribute to disease development independently (heterogeneity) or synergistically (interaction). However, to fully explore the complex interplay among risk factors, it will be required to recruit a large number of samples with a wide spectrum of data types, including but not limited to histopathology, EBV, environmental exposures and genetic variation (for both the EBV and human genomes). Such a large-scale study has not been performed yet, but would be possible in the near future with the constant progress of the biotechnological tools.

Not only are multiple risk factors involved in the pathogenesis of NPC, they participate in a stepwise manner (Fig. 3). First, low-grade dysplastic lesions occur in normal nasopharyngeal epithelium, which could be the result of chromosomal changes (e.g., loss of chromosome region 3p and 9p) that are probably induced by genetic susceptibility and/or environmental carcinogens.[95] The low-grade dysplastic change of the epithelium is hypothesized to predispose the cells to EBV infection, which in turn deregulates a series of cellular pathways and, together with other genetic and epigenetic alterations, results in rapid progression to invasive carcinoma.[95] It is not known specifically what risk factors have taken part in the process, nor is it clear when and how they are involved.

At present, the complex etiology of nasopharyngeal carcinoma is far from completely understood. The complexity of the disease requires that researchers from different fields collaborate to bring a multidisciplinary focus to the problem. Above all, we must ensure that knowledge derived from the studies is translated into public health measures within the large general at-risk population, particularly in endemic areas where NPC is a major public health problem.

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CHAPTER 4

EPSTEIN-BARR VIRUS AND THE PATHOGENESIS OF NASOPHARYNGEAL CARCINOMAS

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Abstract: The concept that Epstein-Barr Virus (EBV) is one etiological factor of NPC is supported by multiple clinical and experimental observations including the consistency of the association and the demonstration of the oncogenic potential of viral products contained in most tumors. There is growing evidence supporting a scenario of mutual virus-cell interactions in malignant NPC cells: viral products cooperate with cellular factors to sustain the malignant phenotype whereas specific intra-cellular metabolic and signalling conditions contribute to the inhibition of the viral lytic cycle. Untranslated small viral RNAs are suspected to exert substantial oncogenic effects. The EBERs which interact with cellular receptors of double-strand RNAs have the capacity to block interferon pathways and to stimulate IGF-1 production by epithelial cells. A number of microRNAs are transcribed from the viral genome for which cellular targets are under investigation. Some of them have the capacity to block the expression of cellular pro-apoptotic proteins like PUMA and Bim1. Viral membrane proteins—LMP1 and LMP2—activate multiple signalling pathways, especially the PI-3-kinase pathways and a unique NF-κB pathway which depends on nuclear translocation of a Bcl3/p50/p50 complex. EBNA1 which is required for the maintenance of the viral genome in proliferating malignant cells probably also has direct oncogenic effects especially through the disruption of PML nuclear bodies. BARF1 is another viral protein which is abundantly secreted by NPC cells; it is suspected to contribute to their abnormal proliferation and local immune suppression. Investigations on the premalignant nasopharyngeal mucosa suggest that alterations of the cellular genome—especially DNA losses in the 3p and 9p chromosomes—occur early, sometimes several decades prior to tumor development. Establishment of latent EBV infection in some of these premalignant cells probably results in a high risk of rapid progression from one infected cell towards a monoclonal invasive tumor. Multiple genetic variations are found in EBV isolates. Whether some

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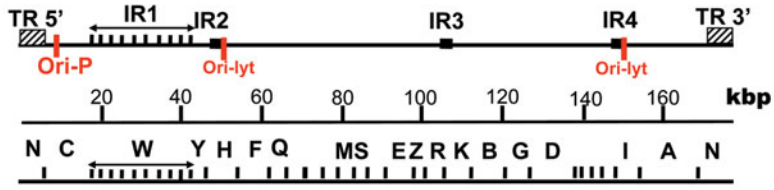
genetic variants of EBV carry a higher risk of NPC is still under investigation. The continued expression of viral products in NPC cells provides multiple opportunities for immuno-therapy, use of inhibitors targeting critical activated pathways and/or specific molecular therapy directed toward the viral functions.

INTRODUCTION

The EBV-NPC relationship was first suspected on the basis of serological observations by Old et al in 1966.[1] It was formally demonstrated a few years later by in situ hybridization of the viral DNA in the nuclei of epithelial cells.[2] The full length EBV-genome is contained in all malignant epithelial cells but not in most infiltrating lymphocytes. The association with EBV is constant—regardless of patient geographic origin—for the nonkeratinizing forms according to the 2005 WHO classification (Types II and III of the 1978 classification).[3,4] NPC oncogenesis is not simply a consequence of systemic EBV infection, as more than 95% adults in all ethnic groups through the world are healthy carriers of EBV.[5] Its occurrence in a restricted subset of infected individuals suggests that there are contributing factors and perhaps unique aspects of infection in those who develop cancers. As stressed in many chapters of this book, NPC is a multifactor disease resulting from specific interactions between environmental, genetic and viral factors. EBV infection is one major etiological factor although not the unique factor.

GENERAL CHARACTERISTICS OF THE EPSTEIN-BARR VIRUS

Epstein-Barr virus (EBV) is a human herpesvirus with a dual lymphoid and epithelial tropism. Like other herpesviruses, it is a double-strand DNA enveloped virus with a large genome (approximately 180 kb with about 80 open reading frames).[6] More details about the structure of the viral genome are provided in Figure 1. One of the most striking and constant pathological characteristics of herpesviruses to produce lifelong infections regardless of the host species. Once a subject has been primo-infected, he will remain infected until death, generally as a healthy carrier. The ability of herpesviruses to produce cellular latent infection in a specific anatomical site is essential for their long term persistence. Latent infection is characterized by the inhabitation of the viral genome in the infected cells with restricted expression of a few viral genes and absence of viral particle production. There are several remarkable features of latent infection by EBV. First, in latently infected cells, circular copies of the viral genome (1 to several hundreds) coexist with cellular chromosomal DNA.[7,8] These circular viral genomes coated by nucleosomes are called episomes. They undergo a process of passive replication and balanced segregation at each mitosis which allow their persistence in an expanding cell population. To a large extent, the viral genome is silenced in latently infected host cells but not completely. Depending on host cell differentiation and metabolic conditions, there is expression of various sets of viral genes encoding viral products called “latent products”. These products are either proteins or noncoding RNAs most of them with the potential to contribute to apoptosis resistance or proliferation of the host cells. Latent proteins are either nuclear (called Epstein-Barr nuclear antigens: EBNA1, EBNA2, EBNA 3a,b,c, EBNA-LP) or associated to the cell membrane network (called latent membrane proteins : LMP1, LMP2 a and b).[5]



EBV products	Coding region	Examples of proposed functions in NPC cells
EBER 1 and *2*	BamH1 C	Non-coding RNAs - Inhibit the RNA-dependent protein kinase (PKR) - Activate the Toll-like receptor 3
BART microRNAs	Bam H1 A	Inhibit expression of some viral lytic and cellular pro-apoptotic genes
LMP1	BamH1 N	Activator of the Bcl3/p50/P50 NF-κB complex
LMP2A	BamH1 N	Activator of the PI3 kinase/Akt pathway
LMP2B	BamH1 N	Accelerate the degradation of interferon receptors
EBNA1	BamH1 K	Episome maintenance – Contributes to disruption of PML bodies
BARF1	BamH1 A	Secreted protein - Ligand of the m-CSF or CSF1 receptor

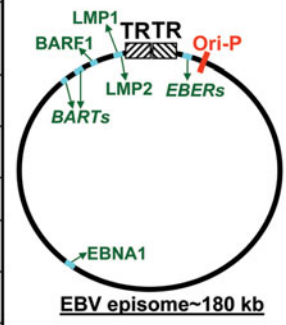


Figure 1. EBV genome structure and mapping of the viral products consistently expressed in NPC. Upper panel: The EBV genome contains about 180 kilobase pairs (kbp). It has a linear form when it is contained in the virions or following replication by the viral DNA-polymerase in cells undergoing a lytic/productive cycle. Both ends of the linear DNA contain a variable number of tandem direct repeats of 500-base-pair sequence, designated terminal repeats (TR) which facilitate the circularization of the viral DNA in latently infected cells. A variable number of direct tandem repeats of about 3000 pb are found in a region called *IR1* (internal repeat 1). Three smaller internal repeats are designated *IR2*, *IR3* and *IR4*.[158] When the viral genome is replicated in latently infected cells, the cellular DNA polymerase starts replication from a region called *Ori P*. In contrast, the lytic replication of the viral genome involves the viral DNA polymerase and a bi-partite origin of replication called *Ori-lyt*.[159] The two genomic segments constitutive of *Ori-lyt* map just 5' to the *IR2* and *IR4* respectively. The nomenclature of the EBV genome open reading frames is based on the digestion of the viral DNA by the BamH1 restriction enzyme which yields about 26 fragments which are classified by decreasing size order from A to Z. *BARF1* designate the first 5' rightward open reading frame of the Bam H1 A region (Bam H1 A open Reading Frame 1). The corresponding protein has the same designation. This is not always the case. The EBNA1 protein is encoded by the *BKRF1* open reading frame. The *IR1, 2* and *3* match the Bam W, H and K fragments, respectively. The *IR4* match the Bam I region; it is lacking in the B95-8 EBV isolate.[34] Lower panel: The main EBV products consistently expressed in NPC are presented in the left side table with the schematic location of the corresponding genes on the right side. The viral genome is presented under its circular form. This circular or episomal form is maintained in the nuclei of latently infected cells as a replicative unit distinct from cellular chromosomes.

In contrast to latent infection, lytic/productive infection results in the release of a large number of viral particles and death of the host cell; it is supported by production of a wide array of viral proteins, many of them being immunogenic. Productive/lytic infection occurs either directly after penetration of the virion in the infected cell or following a period of latent infection (Fig. 2). Direct productive/lytic infection seems to be more frequent in epithelial cells, especially in salivary glands and tonsils.[9-12] In

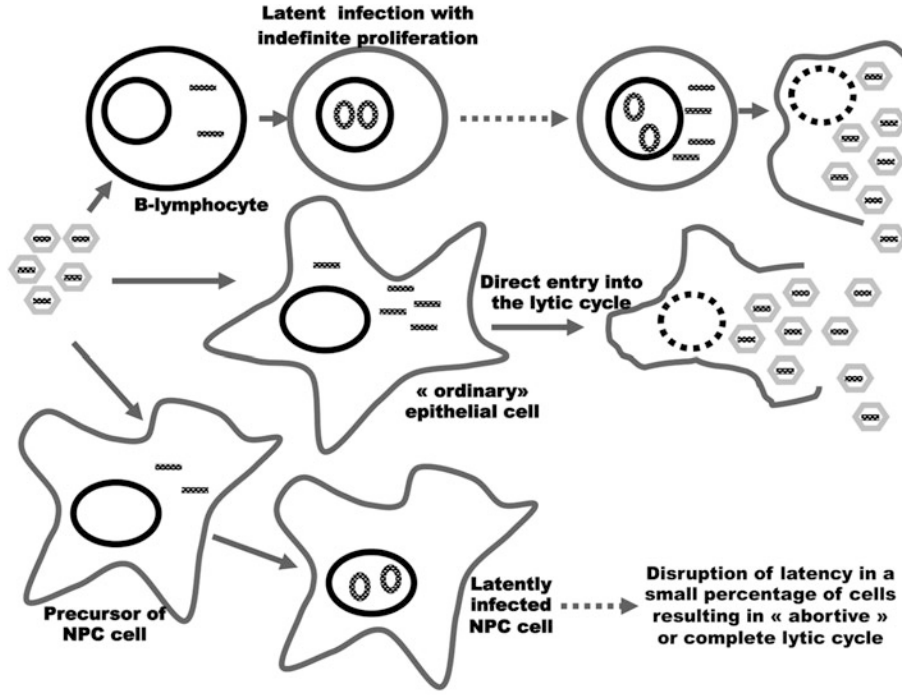


Figure 2. Summary of virus-cell interactions in lymphoid and epithelial cells. In the upper panel, the cartoon depicts resting B-lymphocytes infected by EBV in vitro which undergo EBV-driven transformation with indefinite proliferation. In a small percentage of these EBV-infected B-lymphocytes, the virus-cell balance can switch from latency to lytic/productive infection which is always accompanied by cell death. The middle panel depicts in vitro EBV–infection of epithelial cells which most often results in a direct entry into the lytic/productive cycle. The lower panel illustrates the hypothesis of a peculiar behaviour of the precursors of NPC cells. These cells are supposed to have a phenotype facilitating the establishment of an EBV latent infection. Pre-existing overexpression of cyclin D1 or inhibition of CDKN2A can contribute to this phenotype. EBV latency is often disrupted in a small percentage of malignant NPC cells. The EBV genome is linear in the viral particles. It circularizes in the nuclei of latently infected cells forming one or several episomes. Reappearance of linear EBV-genomes in latently infected cells is one sign of secondary entry in the lytic cycle.

latently infected B cells, the switch from latent to lytic infection often happens when they differentiate in plasmocytes or undergo endoplasmic reticulum stress.[13-15] While in latent infection, EBV DNA persists as an extrachromosomal episome, in lytic infection the episome is converted into replicative intermediates that are cleaved into linear DNA that is packaged into virions.[16]

Long term persistence of EBV in healthy carriers is thought to rely on both restricted latent infection of a subset of memory B cells and periodical bursts of virion production from differentiated B cells. These bursts of virion production have multiple consequences, for example infection of epithelial cells in salivary gland and release of virions in the saliva, recruitment of novel latently infected memory B cells and immune stimulation which results on a negative feed-back on viral reactivations. Presence of EBV in the saliva of healthy carriers is important for its dissemination among human beings.[10]

EVIDENCE OF THE ETIOLOGICAL ROLE OF EBV IN NPC

As stated previously, more than 95% of the world's population is infected by EBV.[5] In the vast majority of the cases, primo-infection by EBV occurs before the age of ten and is completely unapparent. However, in populations with high standards of living and hygiene, the primo-infection can be delayed, occurring in teen-agers or young adults. In these cases, it can be symptomatic resulting in an acute disease called Infectious Mononucleosis.[17] Fortunately, life-long EBV-infection is completely asymptomatic in most human beings who remain healthy carriers. However EBV is linked to the development of specific cancers.[5]

Currently, there is a very strong convergence of clinical, epidemiological and experimental arguments in favour of the etiological role of EBV in NPC. The first argument is the consistency of association. As already stated, EBV-association is subject to no exceptions for the nonkeratinizing forms of NPCs in endemic as well as in non-endemic areas.[3,4] A second argument is the fact that most—if not all EBV products—detected in NPC cells have oncogenic activity in at least one experimental system as explained in the following sections of this chapter. Another argument is the precession of modifications in serum antibodies specific of EBV proteins prior to the onset of invasive NPCs.[18,19] A fourth argument is based on the evidence of unique EBV-isolates contained in the tumor cells. For a given individual, tumor viral isolates are distinct from the ones detected in other anatomical sites. They carry genetic polymorphisms which, according to computational predictions, selectively invalidate EBV-specific CTL-epitopes restricted in the HLA alleles of the patient.[20] This apparent selection of more stealthy EBV isolates in the tumor suggests that viral products are mandatory for tumor development and are expressed in spite of the pressure of the immune system.

OVERVIEW OF VIRUS-CELL INTERACTIONS IN MALIGNANT NPC CELLS

EBV-infection in NPC is mainly latent. So far, EBV-particles have never been detected in NPC tissue sections by electron microscopy. The presence of the viral episomes in the nuclei of all malignant cells is the most reliable indicator of EBV involvement.[2,21] The episome number varies from one to several tens.[7,22] NPC EBV DNA can be detected by several methods, for example in situ DNA hybridization or Southern hybridization following tumor DNA extraction and restriction digestion. This last method is the basis of the classical "Terminal Repeat" assay which demonstrates the clonality of the EBV genomes in NPC tumors.[23] In the nuclei of latently infected NPC cells, EBV episomes coexist, at least in some cases, with viral DNA copies integrated in the cellular chromosomes.[24]

As previously explained, the characteristics of EBV-gene expression are strongly dependent on the host cell differentiation and metabolic conditions. The sets of viral genes expressed in latently infected NPC cells are distinct from the set of genes commonly expressed in lymphoblastoid cell lines (LCL) which result from the transformation of normal donor lymphocytes infected in vitro by EBV. Genes coding for untranslated RNAs called EBERs and for the EBNA1 protein are consistently transcribed in NPC specimens. In contrast, the genes encoding the EBNA2 and 3 are consistently silent in NPC cells.[25-29] The LMP1 and 2 are often expressed in NPC cells although with great heterogeneity from one tumor to another and among the malignant cells of a given tumor.[30-32]

These patterns of expression are characteristic of latency I (EBNA1 + EBERs) and latency II (EBNA1 + EBERs + LMP1) which are also observed in Burkitt's and Hodgkin lymphoma, respectively.[5] Additional characteristics of EBV-gene expression in NPC is the intense production of rightward transcripts through the BamH1 A restriction fragment of the EBV genome.[33,34] These transcripts called BARTs are presented in the next section of this chapter. Further 3' to the BART coding region is an open reading frame called BARF1 encoding a protein which is consistently detected in NPCspecimens.[35,36]

Another important aspect of virus-cell interactions in malignant NPC cells is the role of cellular regulatory factors required to accommodate EBV latent infection. Recently, investigations based on epithelial cells derived from the nonmalignant nasopharyngeal mucosa have drawn attention to the importance of blocking the mechanisms of senescence for successful establishment of stable latent EBV infection[37] (Tsao GSW—talk in Penang—June 2011—to be published soon).

Finally, it is known that latency is consistently disrupted in a small subset of NPC cells resulting in full lytic-productive infection or at least in partial "abortive" lytic infection.[38-40] There is growing evidence that these events contribute to the overall tumor development (see section, *Contribution of Other EBV Proteins to NPC Development*, in this chapter).

ONCOGENIC ROLE OF UNTRANSLATED SMALL VIRAL RNAs

The most abundant viral RNAs in NPC cells are small nuclear untranslated RNAs transcribed by the RNA polymerase III. They are called EBERs 1 and 2 (Epstein-Barr encoded RNA) and contain 166 and 172 nucleotides respectively. The number of EBER copies per cell can amount to more than 10^6 per cells.[29] EBER expression is constant in NPC although sometimes with unequal concentration among malignant cells in a given tumor.[41] They are strongly bound to ribonucleoproteins particles.[42] For this reason, the EBERs are quite stable in tumor cells and are readily detected in paraffin-embedded tissue sections by in situ hybridization.[29]

Evidence of the oncogenic role of the EBERs has recently accumulated. One major step has been the understanding that their 3D folding creates double-strand RNA structures. These structures interact with at least 3 types of intracellular receptors for double-strand RNAs : the PKR (protein kinase RNA-dependent), RIG1 (retinoic acid–inducible gene-like receptor 1) and TLR3 (Toll-like receptor 3).[43] PKR is an interferon-inducible nuclear kinase whose activation is a critical step in the arrest of protein synthesis induced by the interferons (the eIF2α factor is one of its substrate). The EBERs block PKR activation allowing protein synthesis to proceed even under stimulation by the interferons.[43] Although most EBER copies are concentrated in the nucleus, a significant fraction of them reach the cytoplasm and even the extra-cellular space.[43] Cytoplasmic and extra-cellular EBERs interact with RIG1 and TLR3.[44] In the epithelial cells, the EBERs induce the production and release of the insulin growth factor 1 (IGF1) which is an autocrine growth factor for this category of cells.[45] It is not yet known to what extent RIG1 and TLR3 are involded in this process. However there is indirect evidence of a constitutive endogenous stimulation of TLR3 in NPC cells (Friboulet et al 2008).

A family of rightward transcripts from the BamHI A region was initially identified in cDNA libraries from NPC xenografts.[33,34] One of their functions has been recently elucidated as they appear as a major source of viral micro-RNAs called miR-BARTs.[46-48] Overall, at least 25 species of BART microRNAs can be detected in NPC cells. They can

inhibit at least 3 categories of target genes. The first category comprises viral genes encoding effectors of the lytic cycle. For example miR-BART2 down-regulates expression of the viral DNA polymerase gene.[49] The second category of miR-BARTs target viral genes encoding latent viral products especially LMP1 and LMP2. For example, miR-BART 22 (cluster 2) inhibits LMP2 expression.[50,51] The third functional category of miR-BARTs suppresses cellular genes encoding pro-apoptotic proteins. MiR-BART5 suppress the p53 up-regulated modulator of apoptosis (PUMA), a pro-apoptotic protein belonging to the BH3-only class of the Bcl-2 family and inhibit apoptosis.[52] Several miR-BARTs cooperate in the inhibition of the expression of the pro-apoptotic protein Bim.[53] It is obvious that this last category has a direct oncogenic effect. Inhibition of the lytic/productive cycle is also important for the long term growth of NPC tumors since malignant cells escaping latency will die or stop their proliferation. Attenuation of LMP1 and LMP2 expression is also critical because several observations suggests that there is only a narrow margin from oncogenic to endogenous cytotoxic effects.[54,55]

CONTRIBUTION OF THE LATENT MEMBRANE PROTEIN 1 TO NPC DEVELOPMENT

LMP1 (or latent membrane protein 1) was the first EBV protein whose oncogenic activity was formally demonstrated.[56] The oncogenic potential of LMP1 in epithelial cells is demonstrated by the observation of multiple phenotypic changes resulting from the transfection of its gene, mostly in previously immortalised or transformed cells. Most remarkable changes include : various alterations of cell morphology, inhibition of differentiation in organotypic cultures, enhancement of clonogenic growth and induction of an epithelio-mesenchymal transition, induction of the expression of growth factor receptors like EGFR or met, induction of transcription factors like Id1, Id2 and HIF1α, transcriptional silencing of CDKN2A (p16/Ink4).[57-65] A recent report indicates that LMP1 and the catalytic subunit of the human telomerase can cooperate to immortalize primary epithelial cells from the nonmalignant nasopharyngeal mucosa.[66] The oncogenic effects of LMP1 are almost constantly associated with pro-inflammatory phenotypic changes, including an increase in the membrane expression of CD40 and CD70, the release of inflammatory cytokines like IL-1 and IL-6 and an increase in the expression of interferon responsive factors.[58,67-70]

LMP I is an integral membrane protein of 386 amino-acids containing a short cytoplasmic amino-terminal portion (residues 2 to 23), a membrane-associated portion with six transmembrane segments (between residues 24 and 184), and a long cytoplasmic carboxy terminal portion (residues 185 to 386) (Fig. 3)[71] (UniProt accession number P03230). There is strong evidence that LMP1 mainly signals from the internal membrane network.[72] It activates an impressive number of intra-cellular signalling pathways, including canonical (p65/p50) and noncanonical (p105/p50) NF-κB cascades, several MAPK kinase pathways (Erk1/2, JNK, p38), STAT3, the PI3-kinase/akt cascade, PERP/eiF2α, PKC δ.[73-80] In addition, it alters calcium exchanges and metabolism with a net increase in the concentration of free cytosolic calcium.[81-83] At least three types of molecular events are critical for the activation of some of these signalling pathways: the self-aggregation of LMP1 through its transmembrane domains, its incorporation into small membrane domains called membrane rafts and the recruitment of cellular signalling adaptors by its C-terminal intra-cytoplasmic domains.[80,84-88] Most of these adaptors are

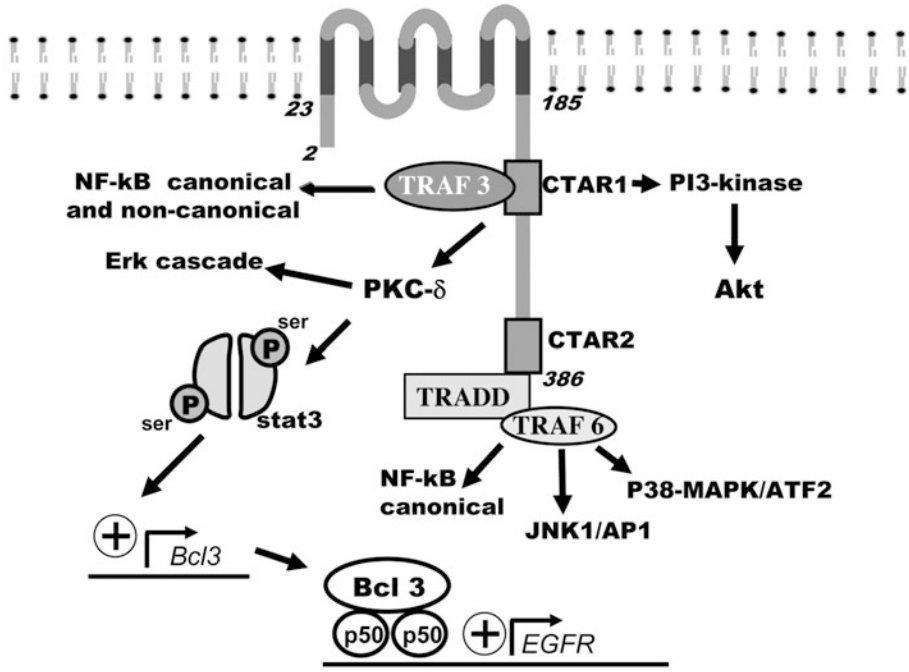


Figure 3. Functional map of the LMP1oncoprotein and the mechanisms of its contribution to the EGFR expression. LMP1 (latent membrane protein 1) is made of 386 amino-acids. Italic numbers indicate the limits of distinct portions of the molecule: the intra-cytoplasmic N-terminus (residues 2-23), the 6 transmembrane segments and connecting loops (residues 24-184) and the intra-cytoplasmic C-terminal portion (residues 185-386). The C-terminal portion contains 2 signalling domains CTAR1 (194-232) and CTAR2 (351-386)(C-terminal activating regions 1 and 2).The TRAF3 protein is the main cellular partner of CTAR1.[89,90] TRADD and TRAF2 are two partners of CTAR2 which can activate at least 3 signalling pathways, the canonical NF-κB, JNK/AP1 and p38-MAPK/ATF2 pathways.[75,160] The contribution of these pathways to LMP1 signalling in NPC cells is not known. CTAR1 can activate the NF-κB canonical and noncanonical pathways, the PI3-kinase, Erk and Stat3 pathways.[73,78,79,161] In transfected epithelial cells, LMP1 activates a peculiar Bcl3/p50/p50 NF-κB activation complex which is also detected in malignant NPC cells in situ.[91,92] Upstream of this complex, LMP1 activates the PKC-δ which phosphorylates Stat3. PKC-δ also activates an Erk cascade.[78] Activated Stat3 stimulates the transcription of *Bcl3* whose protein binds a p50-homodimer (p50 is derived from the NF-κB1 p105). The resulting BCL3/p50/p50 complex activates the transcription of *EGFR*.[91,92] The mechanisms of formation of the p50 homodimers and their translocation to the nucleus are not fully understood although there is evidence that these steps require TRAF3 binding to CTAR1.[90,162]

physiological partners of the TNFR-family of membrane receptors. The most important of them is TRAF3.[89,90]

In experimental systems, the induction of EGFR gene transcription by LMP1 is based on its capacity to induce the formation of a unique type of NF-κB activation complex containing the Bcl3 protein and 2 NF-κB1 p50 subunits (Bcl3/p50/p50). The formation of this complex is highly dependent on the STAT3 pathway with activation of the PKCδ operating upstream of STAT3[78,91] (Fig. 3). Interestingly, unusual Bcl3/ p50/p50 complexes are detected in NPC xenografts and chromatin immunoprecipitation demonstrates that they are bound to NF-κB sites within the EGFR gene promoter.[92]

For a long time it has been assumed that LMP1 was present in about 40% NPC biopsies.[25,93] However using more sensitive methods for immunohistochemistry—for example tyramine-enhancement—it has been possible to detect the LMP1 protein in almost 100% of NPC biopsies; a finding which is consistent with the data obtained by reverse PCR using nested primers.[28,30,94,95] It remains that the amount of LMP1 is highly variable from one biopsy to another and highly heterogeneous among the malignant cells of a given biopsy.[30] Surprisingly, biological features potentially related to LMP1 action—for example expression of EGFR, Bcl3, Id1, CD40 or CD70—are not significantly different in NPC biopsies where LMP1 is either abundant or beyond the threshold of detection.[92,93,95-97] There are several ways to explain this paradox. First, a recent study has shown that LMP1 is in reality produced by an apparently LMP1-negative NPC cell line but is rapidly degraded by the proteasome (it becomes detectable when cells are treated with a proteasome inhibitor).[98] Secondly, we know that LMP1 can significantly alter the phenotype of host cells at a very low concentration even below the threshold of detection by immuno-histochemistry.[99] Thirdly, we suspect that nanovesicles called "exosomes" have the capacity to redistribute LMP1 from a few high producer cells to numerous bystander malignant or stromal cells.[100,101] Finally, other factors might substitute to LMP1 in truly negative cells, for example factors inducing an endogenous activation of NF-κB. The constitutive loss of IkBα expression in an NPC xenograft where LMP1 is apparently undetectable is consistent with this hypothesis.[85,92]

CONTRIBUTION OF THE LATENT MEMBRANE PROTEIN 2 TO NPC DEVELOPMENT

The latent membrane proteins 2 A and B (LMP2 A and B) are encoded by highly spliced mRNAs that contain exons located at both ends of the linear EBV genome.[102] The two forms of LMP2—LMP2A (P13285-1 in UniProt) and 2B (P13285-2)- differ in that only LMP2A has a 119 amino acid N-terminal cytoplasmic domain. LMP2A has 497 amino-acids with three main portions: a cytoplasmic N-terminal domain (residues 1-123), a membrane-associated portion with 12 transmembrane segments (residues 124-470) and a short C-terminal cytoplasmic domain (residues 471-497). The N-terminal cytoplasmic domain contains several tyrosine residues which can be phosphorylated and are essential for the function of this domain which is the "signalling arm" of the LMP2A.[103]

The LMP2A does not transform primary murine fibroblasts or epithelial cells on its own but it induces remarkable changes in the phenotype of epithelial cells: enhancement in growth properties in vitro and in xenografts, inhibition of differentiation, epithelio-mesenchymal transition, switch to a phenotype of cancer initiating cells (or cancer stem cells), increase in the capacity of migration and invasion, nuclear translocation of the β-catenin.[32,103-108] Many of these effects are supported by activation of the PI3K/Akt pathway.[103] In some epithelial cell backgrounds, activation of PI3K/Akt by LMP2A requires up-stream activation of the ras proteins.[108] LMP2A induces Δp63 expression which contributes to the alteration of epithelial maturation.[109] So far, only few studies have addressed the biological effects of LMP2B in epithelial cells. There is evidence that it can enhance motility of epithelial cells independently of the activation of the PI3K/Akt pathway.[106] Both LMP2 A and 2B have also been reported to accelerate the degradation of interferon receptors.[110]

Using highly sensitive methods of immunohistochemistry, LMP2A is detected in about 50% of NPC biopsies.[31,32] Interestingly, LMP2A is more abundant at the "invasive front", at the periphery of the tumor where malignant cells are in contact with healthy tissues.[32]

CONTRIBUTION OF OTHER EBV PROTEINS TO NPC DEVELOPMENT

EBNA1 is the unique EBV nuclear protein known to be consistently expressed in NPC cells and the first viral protein which has been detected in this tumor.[111,112] Its expression is relatively homogeneous in all malignant cells in a given tumor.[113,114] EBNA1 is absolutely required in proliferating latently infected cells as a critical factor for the replication of the viral episomes and their balanced segregation in dividing cells (mitotic segregation).[115] In this respect, EBNA1 is indirectly oncogenic as a necessary factor of EBV genome maintenance in an expanding tumor population. In addition there is growing evidence that EBNA1 also has signalling activity and direct oncogenic effects, especially in NPC cells.[116]

EBNA1 has 641 amino-acids forming several functional domains including a Gly/Ala repeat (residues 90-325), critical domains for mitotic segregation and DNA binding (residues 325-376 and 459-607 respectively) (UniProt accession P03211).[117,118] To perform its episome maintenance functions, EBNA1 needs to bind the latent origin of replication of the EBV genome (*Ori P*, bases 7315 to 9312). In addition to its role in the maintenance of EBV episomes, EBNA1 modulates expression of some viral transcripts.[119] It is also suspected to influence the transcription of some cellular genes, for example *STAT1* but this is still debated.[120,121]

Finally EBNA1 has signalling activity through protein interactions especially with USP7 (ubiquitin specific protease 7). USP7 is a key partner of p53 which prevents its ubiquitylation and subsequent degradation by the proteasome. EBNA1 competes with p53 to bind to the same pocket of the USP7 protein and it blocks p53 increase in UV-irradiated U2OS (an osteosarcoma cell line which retains wild-type p53).[122] These data suggest that EBNA1 might contribute to functional inactivation of wild-type p53 in NPC cells.[123] However, it does not explain why malignant NPC cells often contain high amounts of wild type p53.[124] EBNA1 has also been shown to induce disruption of promyelocytic nuclear bodies (PML-NB) in NPC cells.[116] These nuclear structures are important for DNA repair and apoptosis after DNA damage. Their alteration by EBNA1 is likely to increase genetic instability in NPC cells. While EBNA1 is antagonist of USP7 with regard to stabilisation of p53, it seems to cooperate with USP 7 to induce disruption of the PML-bodies.[125]

BARF1 is an EBV protein named according to its coding open reading frame (Bam H1 Reading Frame 1). Along with LMP1, it is the only protein which is known to transform rodent fibroblasts.[126] BARF1 also has oncogenic activity in epithelial cells. Transfection of the BARF1 gene immortalizes monkey kidney primary epithelial cells.[127] It has also anti-apototic effects in various types of epithelial backgrounds.[128,129] BARF1 has 221 amino-acids and undergo N-glycosylation on the asparagine 95 residue (UniProt P03228).[130] The BARF1 protein is a secreted protein which is not easily detected in cell lysates.[131] Extra-cellular BARF1 is highly soluble and forms a stable hexamer.[132] BARF1 has strong homology with the fms-receptor and binds the m-CSF (colony stimulating factor 1).[133] M-CSF is a hematopoietic growth factor required for monocyte/macrophage maturation which is the natural ligand of the c-fms receptor. Consistently, BARF1

inhibits production of interferon-α by human monocytes.[134] Soluble BARF1 has been reported to enhance in vitro the proliferation of eucaryotic cells especially fibroblasts and epithelial cells.[129,135] However, these data have not been reproduced by other groups. BARF1 mRNAs are detected by RT-PCR in most NPC specimens. The protein is detected by immunohistochemistry and/or western blot in about 80% of the cases.[136] Detection of soluble BARF1 in plasma samples has been reported in the vast majority of NPC patients.[137] In brief BARF1 is an EBV oncoprotein and probably a decoy receptor for the m-CSF. The role of soluble BARF1 in NPC tumor growth needs further assessment.

As previously mentioned in this chapter, EBV-latency is breached in a small subset of malignant NPC cells resulting in a complete lytic/productive cycle or at least in a so-called abortive lytic cycle. Occurrence of the lytic cycle is attested not only by the detection of linear forms of the genome in NPC biopsies but also by the detection of viral proteins of the lytic cycle in a small minority of cells, for example the immediate early protein ZEBRA (BZLF1), the viral DNase (BALF5) and the main envelope glycoprotein gp350 (BLLF1).[23,38-40] Scattered cells producing lytic proteins can be visualized in a majority of NPC biopsies most frequently in malignant cells with intermediate epithelial maturation.[40] For a long time, disruption of latency has only been regarded as a limiting factor of oncogenesis. Indeed cells entering the lytic cycle are expected to die or at least to be unable to proliferate. However recent studies suggest that these cells can also contribute to tumor development by indirect means especially secretion of cytokines which enhance malignant cell proliferation, angiogenesis or local immunosuppression.[138,139] For example, induction of the lytic cycle in EBV-converted epithelial cells stimulates the production of IL-8.[139] Occurrence of the lytic cycle in a minority of malignant cells also explains the rise of circulating antibodies against early and late antigens in NPC patients.[18,19]

EBV AND MULTISTEP CARCINOGENESIS OF NPC

NPCs generally occur several decades after EBV primo-infection. However, in contrast with the oncogenesis of cervical carcinomas associated to HPV, there is no evidence of a long precession of the viral infection in premalignant mucosal lesions. First, it is rare to observe morphological alterations of the nasopharyngeal mucosa truly suggestive of a premalignant state. In a landmark study, Pathmanathan et al (1995) have screened 5326 archival nasopharyngeal biopsies to finally select 11 cases of pre-invasive lesions (dysplasia or carcinomas in situ) without adjacent invasive carcinomas! Remarkably these lesions were containing EBNAI, LMP1, LMP2A and BART transcripts. Moreover, in 6 samples available for tissue sectioning, all cells were staining positive for LMP1, with detection of the EBERs in the majority of them.[140] Later on, complementary information was provided by studies based on systematic search of genetic alterations in small fragments of the nasopharyngeal mucosa obtained from individuals living either in endemic or non-endemic areas. In many of these specimens, the mucosa was exempt of morphological alterations.[141,142] These investigations have revealed frequent allelic losses of chromosomes 3p and 9p in normal-looking mucosa samples from endemic areas (southern China) but not from non-endemic areas (northern China and Canada). In some cases, distinct molecular alterations were found in distinct fragments of the mucosa from the same individual.[143] This is in contrast with invasive NPCs where these allelic deletions are quite common but display a monoclonal pattern. Remarkably, no EBV-infection was detected in association with the pretumoral lesions devoid of morphological alterations.

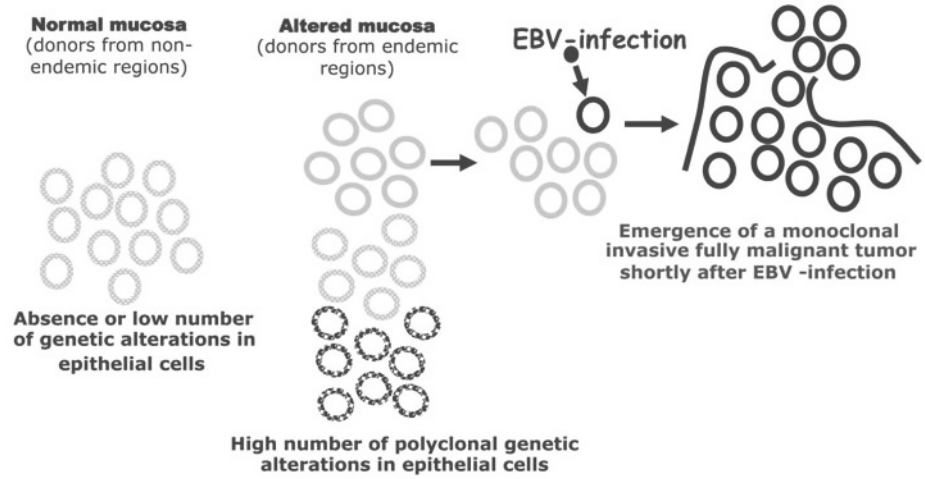


Figure 4. EBV and the multistep carcinogenesis of NPC. Groups of colour circles are symbols of epithelial cell populationsat the surface of the nasopharyngeal mucosa. Only few genetic abnormalities are detected in fragments of mucosa collected in regions which are not endemic for NPC. In contrast, numerous losses of heterozygoty (LOH) affecting chromosomes 9p and 3p are detected in mucosal fragments from endemic regions, even in the absence of morphological abnormalities. The characteristics of these LOH are not identical at various sites of the mucosa, probably as a result of multiple genetic alterations occurring independently in these various sites. EBV DNA or EBERs are not detected in these altered epithelial cells. In fact EBV is rarely detected before the onset of an invasive tumor growing across the basal membrane of the epithelium and displaying a monoclonal pattern of 9p and 3p LOH. One possible interpretation of these observations is that as soon as EBV is able to establish a latent infection in an epithelial cell containing previous genetic alterations, this cell becomes rapidly fully malignant.[140-143]

One way to explain these observations is to consider the following sequence of events: (1) first molecular alterations occur in the genome of nasopharyngeal epithelial cells long before the establishment of a latent EBV-infection; (2) EBV particles are periodically released by B cells at proximity of the nasopharyngeal mucosa; (3) rare events lead to the establishment of a latent infection in a clone of epithelial cells which then rapidly progress to full malignancy and become immediately invasive or go through a very transient stage of severe dysplasia or pre-invasive carcinomas (Fig. 4).[143]

EBV STRAIN HETEROGENEITY AND THE ETIOLOGY OF NPC

The endemic patterns of incidence of NPC and other EBV-associated malignancies have prompted studies to discern if there are distinct strains of EBV with distinct biologic properties that might contribute to these differences in disease incidence. Ideally, classification of EBV-isolates would require complete sequencing of their genome. However so far, the full sequence of only 6 EBV-isolates has been published.[144,145] Meanwhile, numerous studies have investigated small groups of polymorphisms affecting EBV-genes coding for latent or lytic proteins like EBNA1, LMP1, LMP2, ZEBRA, BHRF1 or the EBERs.[144,146-150] These studies have revealed a substantial genetic diversity among

EBV isolates. Most viral polymorphisms described so far are strongly dependent on the geographic origin of EBV-carriers as well as the anatomic site where they are collected (for example blood or saliva).[151-153] None of them have a consistent relationship with NPC or another disease state, although there are some trends; for example the LMP1 deletion of 30 pb is more frequent in isolates derived from tumors than from healthy subjects in several areas of Asia, Africa and South America.[144,154] One challenge for the years to come is to perform simultaneous analysis of the classes of polymorphisms from multiple distinct segments along the viral genome in order to achieve a more comprehensive classification of EBV strains.

CONCLUSION

Multiple observations support the assumption that EBV is a critical factor not only for the initial steps of NPC oncogenesis but also for the maintenance of the malignant phenotype. The obligatory expression of various viral products in malignant cells provides multiple opportunities for specific therapeutic targeting. Immunotherapy is one possible approach (see Chapter 11 by Smith and Khanna). Other groups intend to use small-molecule inhibitors targeting viral proteins associated to latency. For example there is ongoing research for small peptides with the capacity to block the EBNA1 binding to its cognate *Ori P* sites on the EBV DNA.[155] Alternatively, it might be possible to achieve therapeutic selectivity by targeting cellular signalling pathways activated by viral products. For example, the constitutive activity of TLR3 (Toll-like receptor 3) in NPC cells—which is probably a consequence of EBER production—is associated with marked vulnerability to a pharmacological inhibitors of the IAPs (Inhbitor of Apoptotis Proteins).[156,157] In another field of research, identification of EBV strains with high oncogenic risks would be of obvious interest to support a policy of long term prevention against NPC.

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CHAPTER 5

ACQUIRED GENETIC AND EPIGENETIC ALTERATIONS IN NASOPHARYNGEAL CARCINOMA

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Abstract: Nasopharyngeal carcinoma (NPC) has a distinct geographic distribution and strong association with Epstein-Barr virus (EBV). Recent advances in molecular investigations and bioinformatics have disclosed critical genetic and epigenetic events in NPC. In this chapter, we will focus on important genetic and epigenetic alterations in NPC derived from EBV positive NPC cell lines and human tumoral tissues. Copy number losses on chromosomes 1p, 3p, 9p, 9q, 11q, 13q, 14q and 16q and recurrent gains on chromosome 1q, 3q, 8q, 12p and 12q were frequently observed in NPC. The roles of several important tumor suppressors (e.g., *p16, RASSF1A*) and oncogenes (e.g., *CCND1, LTβR*) have been delineated. However, potential critical cancer associated genes in other chromosomal regions remain to be identified. Frequent wide-spread methylation of cancer related genes is another common phenomenon in NPC and leads to alterations of multiple cellular pathways. The possible mechanisms of NPC tumorigenesis, in particular the roles of EBV latent gene products, have been suggested. There is also emerging information concerning the disruption of various signaling pathways including NF-κB signaling pathways in NPC. NPC serves as a fascinating model to understand the complex interaction among environmental, viral, and genetic factors in human tumorigenesis. Important genetic and epigenetic alterations in NPC are summarized in this chapter. Based on these observations, a hypothetical model of NPC tumorigenesis is proposed and serves as a platform for continuous refinement.

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is remarkable for its striking geographic and racial distribution. NPC is prevalent in Southern China, Southeast Asia and North Africa. More than 80% of new cases are detected in these endemic regions. In Southern China, the incidence rate is about 25-50 per 100,000 persons per year and is 100-fold higher than that in the Western population.[1,2] The etiology of NPC is multifactorial. Genetic susceptibility and environmental risk factors, including intake of preserved foods (e.g., salted fish) at an early age and Epstein-Barr virus infection, are implicated in NPC tumorigenesis.[3] These factors may directly or indirectly contribute to the acquired genetic and epigenetic alterations that are responsible for initiation and progression of NPC. This distinctive-type of head and neck cancer may serve as an interesting model for molecular carcinogenesis. Recent advances in molecular genetics and bioinformatics have revealed multiple molecular alterations in NPC. In this chapter, we will focus on critical genetic and epigenetic abnormalities in NPC tumorigenesis and their roles in disrupting normal cellular mechanisms and signaling pathways in nasopharyngeal epithelial cells. The possible contribution of environmental and viral factors in inducing somatic genetic changes and transformation of NPC cells are proposed in a new NPC tumorigenesis model.

KARYOTYPIC AND MOLECULAR ANALYSIS OF NPC

According to WHO classification, there are three subtypes of NPC: Type I (keratinizing squamous cell carcinoma), Type II (nonkeratinizing carcinoma) and Type III (undifferentiated carcinoma). In endemic regions, majority of NPC are Type III and II and these subtypes show consistent association with Epstein-Barr virus infection. EBV infection is present in virtually all cancer cells. The monoclonal nature of EBV genome in invasive carcinoma implies that EBV latent infection may occur prior to the expansion of the malignant clone.[4,5] Expression of EBV latent genes might be critical for initiation and progression of NPC through interacting cellular molecules or directly inducing genetic and epigenetic changes. Therefore, in this chapter, we will focus on the findings reported in EBV-positive NPC tumor lines and primary tumors.

Cytogenetic and molecular alterations in NPC genome have been explored since late 1980s. Cytogenetic information is limited since primary tumors grow poorly in vitro and only a few EBV-positive NPC lines are available. The pioneering cytogenetic works in EBV-positive undifferentiated NPC xenografts from Huang et al[6] and Bernheim et al[7] provided the first piece of information concerning chromosomal abnormalities in NPC. These EBV-positive tumor lines remain important models for molecular and functional analysis for this viral-associated malignant disease. Despite the many complex re-arrangement found, recurrent structural abnormalities on chromosomes 1, 3p, 11q, 12 and 17 were observed.[6-8] Deletion of chromosome 3p was consistently found in NPC cell line, xenografts, and primary tumor biopsies in these studies. Strikingly, the modern molecular cytogenetic and genetic studies have subsequently proven that inactivation of tumor suppressor gene on this chromosomal region is one of most critical molecular events in NPC tumorigenesis.[8-12] By genome-wide screening approaches, including allelotyping/LOH analysis and comparative genome hybridization (CGH), detailed "genome map" for cataloguing genetic alterations in NPC has been established (Fig. 1). A number of recurrent chromosomal abnormalities identified by CGH studies suggested

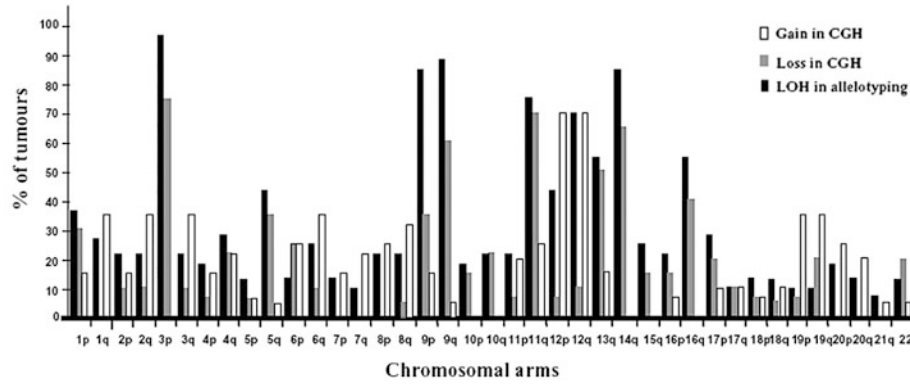

Figure 1. Frequencies of LOH, CGH gain and loss in microdissected NPC tumors.

the involvement of multiple genetic defects in NPC tumorigenesis. High incidences of copy number losses were detected on chromosomes 1p, 3p, 9p, 9q, 11q, 13q, 14q and 16q, while recurrent gains on chromosome 1q, 3q, 8q, 12p and 12q were observed in Chinese NPC patients.[8,9,11,12] Interestingly, distinct regions of gain at 11q13 and 12p12-13 were identified in 53% and 59% primary tumors respectively in a study of Taiwan NPC patients.[11] The findings were confirmed in a recent high-resolution array-based CGH analysis.[13] The tree models constructed by multiple sets of CGH data predicted 3p deletion and chromosome 12p gain as important early events.[14,15] By high-resolution allelotyping study, genome abnormalities on similar chromosomal regions were also demonstrated in a panel of microdissected primary NPCs. Moreover, allelic losses on chromosomes 3p, 9p and 14q were consistently detected in more than 85% of primary tumors.[10] Importantly, high incidence of 3p/9p LOH was also found in the precancerous lesions.[16,17] These findings suggested that the inactivation of tumor suppressor genes in these regions is critical events for transformation of nasopharyngeal epithelial cells.

In addition to deciphering the global genetic changes, these CGH and LOH studies have defined multiple minimal regions (e.g., 3p21, 9p21.3, 11q13.3, and 12p13.3) in which a number of candidate NPC-associated tumor suppressor genes and oncogenes were identified (Table 1).

ONCOGENES

Recurrent copy number gain and amplification of chromosomal regions are commonly associated with activation of oncogenes reside in these regions. Our array-based CGH (aCGH) studies have identified several novel amplicons in NPC.[13] Two most common amplicons in NPC were delineated at chromosome 11q13 and 12p12-13. The incidences of copy number gain of 11q13 and 12p12-13 were 57% and 62% respectively. Fine mapping and detail analysis showed that *CCND1* gene within the 11q13 amplicon is amplified and highly expressed in NPC cell lines, xenografts and primary tumors.[13] *CCND1* encodes the cell cycle regulating protein cyclin D1, which interacts with cyclin dependent kinases (CDK4 and CDK6) in G1 to S-phase transition of cell cycle, initiating DNA synthesis. Knockdown of *CCND1* in NPC cell lines by siRNA showed a dramatic

Table 1. Cancer-related genes involved in NPC

	Location	Function	Abnormalities in NPC
Oncogenes			
PIK3CA	3q26.1	Lipid kinase	Gene amplification
Cyclin D1	11q13	Cell cycle progression	Gene amplification, overexpression
Bmi-1	10p11.23	Polycomb protein	Overexpression
Bcl2	18q21.3	Anti-apoptosis	Overexpression
Tumour suppressor genes			
RASSF1A	3p21.3	Cell cycle arrest, signal transduction, cell adhesion, microtubule stability	Promoter methylation, mutation
BLU/ZMYD10	3p21.3	Stress responsive gene	Promoter hypermethlyation
DLEC1	3p21.3	Unknown	Promoter hypermethylation, histone deacetylation
p16	9p21.3	Cell cycle, G1 control	Homozygous deletion, promoter methylation
ARF	9p21.3	Stabilizer of p53 by sequestering MDM2	Homozygous deletion, promoter methylation
TSLC1	11q22-23	Immunoglobulin superfamily cell adhesion molecule, cell-cell interaction	Deletion, methylation
THY1	11q22-23	Cellular adhesion, proliferation, survival, and cytokine/growth factor responses	Deletion, down-regulation

continued on next page

Table 1. Continued

	Location	Function	Abnormalities in NPC
Others			
RIZ1/RDM2	1p36.2	Histone methyltransferase	Promoter hypermethylation
CRBP4	Ip36.2	Retinoid signaling pathway	Promoter hypermethylation
RARB2	3p24	Retinoid signaling pathway	Promoter hypermethylation
CRBP1	3q23	Retinoid signaling pathway	Promoter hypermethylation
TIG1	3q25.32-q25.33	Retinoid signaling pathway	Promoter hypermethylation
UCHL1	4p14	p53 stabilization	Promoter hypermethylation
PCDH10	4q28.3	Cell-cell connection	Promoter hypermethylation
DAB2	5p13	RAS GTPase pathway	Promoter hypermethylation
HIN-1/SCGB3A1	5q35-qter	AKT signaling pathway	Promoter hypermethylation
GADD45G	9q22.1-q22.2	DNA-damage response	Promoter hypermethylation
DAPK1	9q34.1	Mediator of gamma-interferon induced programmed cell death	Promoter hypermethylation
WIF1	12q14.3	Wnt signaling pathway	Promoter hypermethylation
RASAL1	12q23-24	RAS GTPase pathway	Promoter hypermethyoation
CHFR	12q24.3	Mitotic checkpoint control	Promoter hypermethylation
ENDRB	13q22	G protein-coupled receptor, Endothelin-1 signaling	Promoter hypermethylation
CDH1	16q22.1	Calcium dependent cell-cell adhesion glycoprotein	Promoter hypermethylation
IRF8	16q24.1	Response to IFN-gamma stimulation	Promoter hypermethylation
CDH13	16q24.2-q24.3	Calcium dependent cell-cell adhesion glycoprotein	Promoter hypermethylation
RASSF2A	20pter-p12.1	Ras signaling regulation	Promoter hypermethylation

decrease in cell proliferation. This finding supported the critical role of *CCND1* in growth of NPC cells. Progression of cells from G1 to S phase, on the other hand, is blocked by a tumor suppressor, p16, which acts to disrupt the cyclinD1/CDK4/6 complex. Concurrent overexpression of cyclin D1 and downregulation of p16 has been reported in NPC, suggesting an altered cell cycle control in NPC tumorigenesis.

Another highly amplified region in NPC was chromosome 12p12-13. Using high density oligonucleotide aCGH, we have defined a 2.77 MB novel region of gain at 12p13.31.[18] This amplicon is a gene-rich region, harboring more than 10 genes. We found that several genes in this region were overexpressed in an expression array study. Among the overexpressed genes identified, Lymphotoxin-β receptor (*LTβR*) showed the highest expression level. LTβR was overexpressed in 76% primary NPC tumors with 54% showing amplification of *LTβR* gene. LTβR is a member of the tumor necrosis factor receptor (TNFR) family and is activated by two members of the TNF family, LTα1β2 and LIGHT, which then activates multiple downstream signaling pathways including NFκB and c-Jun N-terminal kinase. Activation of NFκB has been demonstrated in NPC cell lines, xenografts and primary tumors.[19-22] Ectopic expression of *LTBR* highly induced NFκB activity in immortalized nasopharyngeal epithelial cells.[18] This indicates a possible involvement of LTβR overexpression in NFκB activation in NPC tumorigenesis.

Chromosome 3q, in addition to chromosome 11q and 12p, is another region showing consistent high copy number gain and amplification.[9,23] *PIK3CA* residing at 3q26.1 was frequently amplified and overexpressed in NPC cell lines and xenografts. *PIK3CA* encodes the 110-kDa catalytic subunit of phosphatidylinositol 3-kinase (PI3K), which coupled with the 85-kDa subunit activates protein tyrosine kinases and generates second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 in turn activates a wide range of downstream targets involved in cell proliferation, survival, membrane trafficking and cytoskeletal re-organization. Despite that activating somatic mutations of *PIK3CA* are common in breast, liver and colon cancer,[24,25] no mutation has been found in NPC.[26] Thus, copy number gain/amplification of *PIK3CA*, instead of gain-of-function mutation, is a common mechanism in NPC tumorigenesis.

Recent evidences suggest that polycomb group (PcG) genes can act as oncogenes, in addition to their epigenetic gene silencing property. Bmi-1 is one of the polycomb group proteins and was first identified to co-operate with c-Myc in murine lymphoma.[27,28] Song et al[29] found that Bmi-1 was overexpressed at both the mRNA and protein level in NPC cell lines. Overexpression of the Bmi-1 protein was further demonstrated in 38.7% primary NPC tumors. The oncogenic potential of Bmi-1 was revealed by its ability to immortalize normal nasopharyngeal epithelial cells (NPEC). Song et al demonstrated that overexpressing Bmi-1 in NPEC by retrovirus transfection could bypass senescence and result in immortalization. More importantly, overexpression of Bmi-1 resulted in down regulation of p16 and up regulation of telomerase activity. Recent study showed that overexpression of telomerase could also lead to immortalization of nasopharyngeal epithelial cells.[30] In Bmi-1 immortalized NPEC, down regulation of p16 leading to hyperphosphorylation of Rb, resulted in an uncontrolled cell growth. EZH2 is another member of the PcG family that is commonly overexpressed in NPC. It mediates several important cellular processes, such as differentiation, response to ROS and DNA repair. Knockdown of EZH2 inhibited cell cycle progression and induced apoptosis. Expression of EZH2 suppressed FOXM1, Bcl-2, and multiple cell cycle regulators, such as c-Myc, CDK4, CDK6, CCND3 and CCNE2 in NPC cells.[31]

Overexpression of Bcl-2 was found in over 60% of NPC tumors.[32-34] *Bcl-2* is located on chromosome 18q21.3. It is commonly activated by translocation into juxtaposition of immunoglobin heavy chain loci at 14q32 in lymphoma and leukemia.[35] In contrast, no structural abnormality of *bcl2* has been reported in NPC. It has been shown that Bcl-2 expression was closely associated with the presence of EBV.[31] Latent membrane protein 1 (LMP1), one of the EBV latent gene product has been shown to up-regulate and co-operate with Bcl-2 to induce epithelial cell transformation.[36] This suggests that the LMP1 expression together with Bcl-2 overexpression may have an important role in the early step of NPC tumorigenesis.

TUMOR SUPPRESSOR GENES

Since allelic losses at 3p and 9p are critical events in multistep tumorigenesis of NPC, identification of the target tumor suppressor genes in these chromosomal regions is important in understanding the molecular basis of this cancer. By LOH and southern blotting analysis, we have delineated a tumor suppressor locus at 9p21.3 in which homozygous deletion was consistently detected in NPC xenografts and 40% of primary tumors.[37,38] The minimal region of homozygous deletion appears to center on *INK4/ARF* locus encoding the *p15* (*INK4b*), *p16* (*INK4a*) and *ARF* tumor suppressor genes (Fig. 2). Except *p15*, inactivation of the *p16* and *ARF* genes by promoter hypermethylation was also commonly found in tumors without 9p21 homozygous deletion.[39,40] Overall, p16 and ARF inactivation was found in 62-86% and 54% of NPC respectively. Loss of these two tumor suppressors may lead to Rb and p53 dysfunction in the cancer cells. The p16 protein is an important cell cycle regulator that inhibits the cell cycle progression from G1 to S phase. *p16* abnormalities are perhaps the most common mechanism for inactivating pRb/cyclin D1/cdk4/p16 cell cycle control pathway in NPC. Loss of functional p16 will lead to Rb phosphorylation and therefore the release of E2F transcription factor, which constitutively activates the S phase genes for DNA synthesis and results in uncontrolled cell proliferation.[41,42] Restoration of *p16* expression in NPC cells induced G0/G1 arrest and suppressed tumorigenicity in vivo.[43] ARF functions as a tumor suppressor by binding to and inactivating the MDM2 protein that negatively regulates p53. Loss of ARF enables MDM2 to counter-act p53 function more efficiently in response to aberrant growth or oncogenic stresses. Since p53 mutation is rare in NPC, inactivating ARF may be a common mechanism for disrupting the functional p53 in this cancer.[44-46] Interestingly, homozygous deletion of *INK4/ARF* locus was observed during the establishment of an immortalized nasopharyngeal epithelial cell line.[30] The findings suggested that genetic and epigenetic inactivation of *p16* and *ARF* are the critical steps for nasopharyngeal epithelial cells transformation.

On the short arm of chromosome 3, multiple tumor suppressor loci have been reported from a variety of human cancers. These regions include 3p12-13 (*ROBO1/DUTT1* gene region), 3p14.2 (*FHIT* gene region), 3p21.1-p21.2 (*DRR1*, *BAP1*, *ARP*), 3p21.3C (centromeric, LUCA), 3p21.3T (telomeric, A20) and 3p24-26 (*VHL* and *RARβ*).[47] However, NPC-associated tumor suppressor(s) is believed to be located on 3p21.3C (LUCA) in which high frequency of deletion was consistently found in LOH and CGH studies.[9-12,48,49] In our early study, we have demonstrated high frequencies of genetic and epigenetic alterations of the *RASSF1A* (*Ras Association Domain Family 1A*) tumor suppressor gene, which is located within a 120-kb common homozygous deletion region at 3p21.3, in EBV-positive

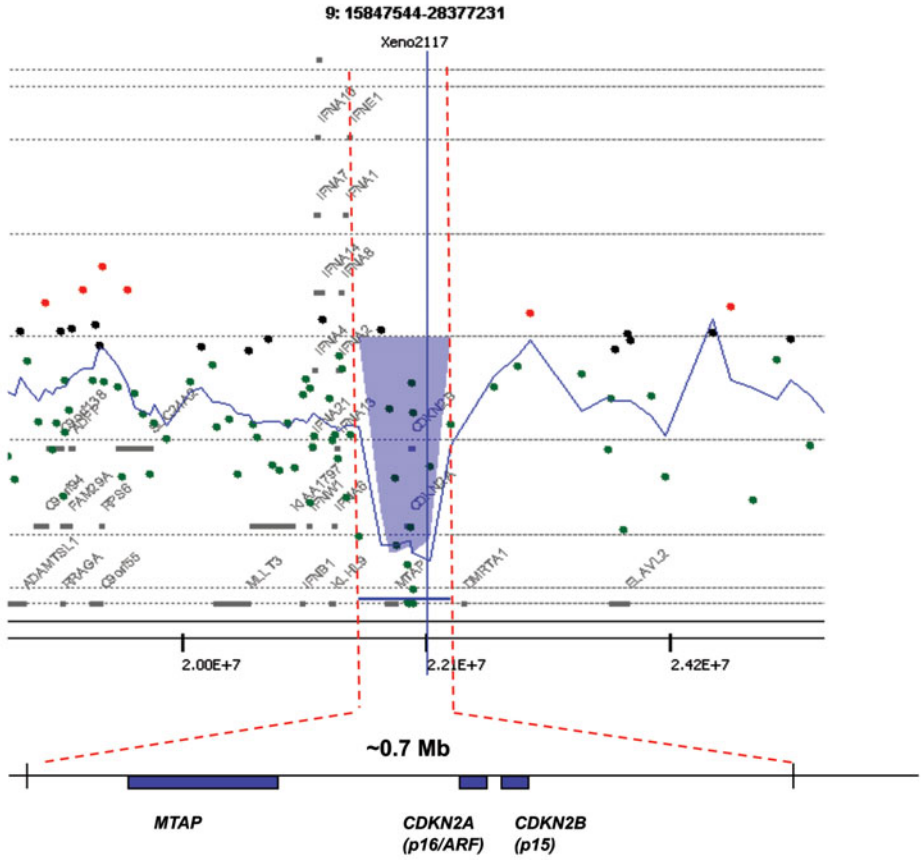

Figure 2. Mapping of homozygous deletion of *INK41ARF* locus at 9p21.3 in a NPC Xenograft (xeno-2117) by high-density array CGH.

NPC cell lines, xenografts and primary tumors.[50,51] Inactivation of *RASSF1A* by promoter hypermethylation is commonly found in a variety of human cancers, including lung cancer, breast cancer, renal cell carcinoma, liver cancer and medulloblastoma.[52-57] In NPC, aberrant methylation and transcription silencing of *RASSF1A* were detected in all EBV-positive xenografts and cell line (4/4, 100%).[50] Aberrant methylation and mutation of *RASSF1A* were also detected in 66.7-83% and 9.5%, respectively, of primary tumors.[50] The presence of missense and silence mutations in primary tumors strengthens the hypothesis that *RASSF1A* is the critical tumor suppressor of NPC. The tumor suppressor function of *RASSF1A* in NPC cells has been demonstrated by transfecting wild-type *RASSF1A* clone in a RASSF1A-deficient NPC cell line. Restoration of wild-type *RASSF1A* led to marked growth inhibition and dramatic reduction in tumorigenic potential of NPC cells.[58] These findings provide functional evidence that *RASSF1A* is a target tumor suppressor gene on 3p21.3 in NPC. RASSF1A is a member of the RASSF family of proteins characterized by a consensus RAS-association domain at the C-terminus. Studies have shown that it may function in the Ras-regulated pro-apoptotic pathway.[59-61] RASSF1A can also inhibit cell

cycle progression by blocking the c-Jun-NH2-kinase pathway and suppressing cyclin D1 accumulation.[62,63] In our recent study, we demonstrated that *RASSF1A* can transcriptionally regulate a number of target genes (*ATF5, TCRB, RGS1, activin betaE, HNRPH1, HNRPD, ID2* and *CKS2*) which are involved in multiple cellular pathways such as transcription, signal transduction, cell adhesion and RNA processing.[64] RASSF1A may function as a tumor suppressor in NPC by repressing ID2 (*inhibitor of DNA binding 2*) expression, whereby its loss leads to epithelial-mesenchymal transition and failure of differentiation. Recently, several groups have reported that RASSF1A is a microtubule-binding protein, which regulates microtubule stability, controls mitotic progression and maintains genomic stability.[61,65-72] As the guardian of mitosis, it regulates APC^{cdc20} activity and ensures the sequential progression of mitosis through direct interaction with Cdc20. Our recent finding suggested that the specific tumor suppressive function of *RASSF1A* in NPC is dependent on the unique N-terminus mediated APC^{cdc20} regulation in mitosis.[73] *RASSF1A*-knockout mice were prone to spontaneous tumorigenesis at advanced age.[74] $RASSF1A^{-/-}$ cells from the knockout mice were much more sensitive to nocodazole induced microtubule destruction than the wild-type cells. By using siRNA targeting RASSF1A, we also found that knockdown of *RASSF1A* in immortalized nasopharyngeal epithelial cells resulted in mitotic failures and enhanced tumorigenic potential. These findings implied that RASSF1A is a major tumor suppressor gene on 3p21.3 in NPC. Aside from RASSF1A, other family members of Ras association domain family (RASSF) are suspected to be candidate tumor suppressors. However, we have previously shown that abnormalities of *RASSF1C*, *NORE1* and *RASSF4* rarely occur in NPC.[58,75] Recently, Zhang et al[76] have reported that promoter hypermethylation of *RASSF2A* was found in 50% primary tumors and correlated with lymph node metastases. Loss of *RASSF2A* in NPC may be beneficial for tumor cell survival by reducing K-ras apoptotic signals.

BLU/ZMYD10 (*zinc finger, MYND-type containing 10*), a candidate 3p21.3 tumor suppressor gene immediately upstream of *RASSF1A*, was also commonly inactivated in NPC. *BLU* is a stress-responsive gene regulated by *E2F* and contains a MYNF domain at its C-terminus. Hypermethylation and downregulation of *BLU* were demonstrated in 66-80% primary tumors.[77-79] Partial methylation of *BLU* was also shown in several EBV-positive xenografts and cell line. Several studies have shown that overexpression of *BLU* led to growth inhibition and tumor suppression in cancer cell lines.[77,80] Although the biological function of BLU is still not known, the MYNF domain at C-terminus is believed to be important for its tumor suppressor activity. It is likely that *BLU* transcription regulates several important target genes involved in cancer development. Further study of the BLU function and its associated pathways is important in understanding the roles of this protein in NPC tumorigenesis. Recently, we have shown that *DLEC1* (*Deleted in Lung and Esophageal Cancer 1*) located at A20 region (3p21.3-3p22.2) is another candidate tumor suppressor gene of NPC.[81] The gene was frequently inactivated in NPC and ovarian cancers by promoter methylation and histone deacetylation.[81,82] Overexpression of DLEC1 suppressed growth, reduced invasiveness, and inhibited tumorigenic potential of cancer cells although its biochemical function is still unclear.

In addition to chromosomes 3p and 9p, two candidate tumor suppressor genes of NPC, *TSLC1* and *THY1*, have been reported at 11q22-23, a region frequently deleted in NPC. *TSLC1* (*tumor suppressor in lung cancer 1*), also known as *IGSF4* (*Immunoglobulin superfamily 4*), encodes an immunoglobulin superfamily cell adhesion molecule (IgCAM), which is a membrane protein involved in cell-cell interactions.[83-85] The protein can directly interact with DAL-1/4.1B and MAGuk to form a ternary complex that participates in

epithelial-like cell structures associated with cell adhesion.[86,87] *TSLC1* may suppress tumor formation by inhibiting epithelial-mesenchymal transition. Loss of its function could lead to invasion or metastasis. The tumor suppression function of TSLC1 has been shown in a variety of cancer cell lines. In NPC, aberrant methylation of *TSLC1* was reported in 34.2% primary tumors.[88] Using tissue microarray and immunohistochemistry analysis, loss of TSLC1 expression were found in 12% of primary NPC and 35% of metastatic tumors.[89] The significantly higher frequency of loss of TSLC1 expression in metastatic tumors suggested that its inactivation might be involved in NPC progression. Apart from *TSLC1*, *THY1* (*Thy-1 cell surface antigen*)/*CD90* on 11q22-23, was also found to be a candidate tumor suppressor of NPC. The protein participates in multiple signaling cascades affecting cellular adhesion, proliferation, survival, and cytokine/growth factor responses.[90,91] A recent study also reported that *THY1* can upregulate thrombospondin-1 and fibronectin, which are associated with cell differentiation and angiogenesis inhibition.[92] Lung et al[93] reported that 40% of primary tumors and 74% metastatic NPCs showed downregulation or loss of THY1 expression in a tissue microarray study. Aberrant methylation may be a possible mechanism for transcriptional silencing of *THY1* in these tumors. These finding suggested that inactivation of *THY1* is involved in lymph node metastasis of NPC.

In addition to the regions mentioned above, inactivation of tumor suppressor gene(s) at chromosome 14q is also believed to be an important event in NPC tumorigenesis since LOH on 14q was detected in more than 85% of primary tumors.[10] However, few candidate tumor suppressor genes for NPC have been identified in this region yet. Searching for the target gene(s) at this chromosomal region may provide further insight in NPC tumorigenesis.

EPIGENETIC ALTERATIONS

For the past decades, epigenetic alterations, including promoter hypermethylation and histone modifications, have been recognized as an important mechanism for the inactivation of cancer-associated genes.[94-96] In NPC, promoter hypermethylation was found to be the major mechanism for inactivation of critical tumor suppressor genes, such as *p16* and *RASSF1A*. Recent studies have detected a widespread hypermethylation of CpG islands of cancer genes over the NPC genome while the contribution of histone modifications in this cancer was rarely reported. The epigenetic changes influenced multiple cellular mechanisms involved in initiation and progression of NPC.

The retinoid signaling pathway in almost all NPC tumors were disrupted by epigenetic inactivation of multiple components including nuclear retinoic acid receptor (*RARB2*), cellular retinoic acid-binding proteins (*CRBP1*, *CRBPV*) and/or retinoid response gene *TIG1*.[40,97,98] The transcriptional silencing of these genes by promoter hypermethylation may result in the loss of cellular retinoic acid homeostasis, inability to uptake natural retinol, and synthesis of retinoic acid. These findings suggested NPC may resist retinoic acid treatment.

Several members of cadherin superfamily, which participate in intercellular and cell-extracellular matrix interactions of cancer, were reported to be the targets for epigenetic inactivation in NPC. E-cadherin (*CDH1*), H-cadherin (*CDH13*), and Protocadherin 10 (*PCDH10*) are methylated in 52%, 89.7% and 82% of primary tumors, respectively.[99-101] Transcription silencing of H-cadherin by promoter methylation was also consistently shown in three EBV-positive tumor lines. Loss of these cell adhesion molecules may

contribute to the progression of NPC by promoting tumor cell invasion and metastasis. Furthermore, inactivation of these genes may be involved in interruption of various cellular functions, including signal transduction, cell growth and differentiation.

High frequencies of promoter hypermethylation in cancer-related genes (*IRF8, GADD45*, *DAPK1, ENDRB, HIN-1, WIF1, RASAL, DAB2, UCHL1*) that are involved in interferon-γ stimulation and DNA damages responses, cell death-signaling network, endothelin-1, AKT, Wnt, Ras GTPase and p53 signaling pathways were reported in several studies.[40,102-109] In addition to *RASSF1A*, the *CHFR* (*checkpoint with forkhead-associated domain (FHA) and RING finger domain*) gene that participates in mitotsis checkpoint regulation is also inactivated by hypermethylation in most of NPC tumor lines and primary tumors.[110] Loss of both *RASSF1A* and *CHFR* may lead to genomic instability. Furthermore, transcriptional silencing of *RIZ1* by promoter hypermethylation may influence the chromatin-mediated gene expression.[111] In comparison with other EBV-negative head and neck cancers, much higher frequencies of promoter hypermethylation in cancer genes were found in EBV-positive NPC. The widespread hypermethylation in the NPC genome implies a methylator phenotype of this EBV-associated cancer. Interestingly, EBV-positive gastric cancer has been reported to show a higher frequency of aberrant methylation than EBV-negative gastric cancer.[112] The observation suggests a relationship between latent EBV infection and epigenetic changes in these EBV-associated epithelial cancers. In NPC, DNA methylation not only contributes to inactivation of cancer genes, it also modifies the Wp and Cp promoters leading to silencing of several EBV latent genes (nuclear antigens EBNA2, 3A, 3B and 3C) and establishment of cell specific type II latency.[113,114] It is likely that epigenetic modification of both viral and cellular genes is crucial in transforming nasopharyngeal epithelial cells. Interestingly, EBV oncoprotein LMP1 was shown to participate in DNA methylation. LMP1 is able to activate cellular DNA methyltransferases via c-jun NH_2-terminal kinase signaling and subsequently induce hypermethylation of several cellular genes, such as E-cadherin.[115,116] On the other hand, LMP1 can upregulate the polycomb group (PcG) protein Bmi-1 which may in turn be responsible for promoter hypermethylation of tumor suppressor genes.[117] The PcG-mediated histone modifications may render certain cancer genes vulnerable to DNA hypermethylation.[118] Thus, latent LMP1 protein expressed in EBV-infected nasopharyngeal cells would induce promoter methylation of several EBV and cellular cancer genes through the upregulation of methyltransferase and PcG protein, and thereby participate in both tumor initiation and progression.

ABERRANT SIGNAL TRANSDUCTION PATHWAYS

In EBV-associated malignancies, the viral latent proteins have been shown to activate multiple signaling pathways and contribute to disease progression. For examples, the LMP1 and LMP2A, which are often expressed in NPC tumors and are known to be able to activate NF-κB, mitogen-activated protein kinase (MAPK), and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways.[119-122] On the other hand, there are also evidences suggesting that genetic and epigenetic alterations are involved in those activating cellular pathways via EBV-independent mechanisms in NPC.

Activating NF-κB signaling pathway is implicated in the development of NPC. Constitutive NF-κB nuclear activity has been consistently demonstrated in EBV-positive NPC cell lines, xenografts and primary tumor.[19-22] There are multiple functions by which

NF-κB can promote transformation and progression of this cancer. Target genes induced by NF-κB are important for controlling cell survival, proliferation, invasion, angiogenesis, innate and acquired immunity. By gene expression profiling, Shi et al[123] have revealed that *NFκB2* (*p100/p52*) and its transcriptional cofactors *RELB* and *BCL3* were significantly upregulated in NPC primary tumors, together with a number of NF-κB target genes, such as *MMP9*, *Bcl-2*, *BFL1*, *BIRC3* and *BIRC5*. Upregulation of other NF-κB target genes (e.g., *VCAM1*, *ICAM1*, *EGFR*, *A20*, *CXCR4*) in NPC has also been reported in several studies.[124-128] It is also possible that abnormal activation of NF-κB is involved in the initial step of transformation. Although NF-κB activity in precancerous lesions is unknown, we have demonstrated that activation of NF-κB was observed in immortalized nasopharyngeal epithelial cell line at later population doublings.[30] These finding suggests the possible role of constitutive NF-κB activation in supporting the growth and survival of immortalized cells. Moreover, activation of NF-κB can inhibit lytic replication of EBV and may therefore contribute to the maintenance of viral latency in nasopharyngeal epithelial cells.[129]

In tumors expressing LMP1, the viral latent protein, NF-κB dimers (e.g., p50/p65, p52/RelB) may be activated through canonical or noncanonical pathways. Interestingly, Thornburg et al[21] has clearly shown that p50/p50 homodimers were specifically activated in NPC while other NF-κB dimers were not detected in the nuclear extracts of both LMP1 expressing and non-expressing NPC cells. The p50/p50 homodimers transcriptional activate downstream targets by binding with Bcl-3 that is overexpressed in most of NPC tumors. Genetic alterations in NF-κB pathways, such as loss of IκB-α and overexpression of Bcl-3, are likely contributing to the abnormal regulation of NF-κB in NPC. Recently, we have found a crosstalk between NF-κB and NOTCH3 signaling pathways. The transcription of *p50/p105* (*NFKB1*) is directly regulated by NICD3 signal which are constitutively activated in NPC cells.[130] Overexpression of NOTCH3 receptor and ligands (DLL4 and JAG1) were detected in almost all EBV-positive tumor lines and primary tumors. We also showed that the activated NICD3 signal is important in maintaining the cancer stem-like cells features, chemoresistance and survival of NPC cells.[130] Dysregulation of NOTCH3 and NF-κB pathways play crucial roles in the development of this EBV-positive epithelial cancer.

By gene expression profiling studies, multiple deregulated signal transduction pathways have been revealed in primary NPCs. The expression microarray showed the differentiated expression of multiple components of WNT/beta-catenin signaling pathway, including two major inhibitors of Wnt/beta-catenin pathway, WIF1 (Wnt inhibitory factor 1, WIF1) and FRZB (secreted Frizzle-related protein 3), which are commonly inactivated in human cancers by promoter methylation.[123] Loss of these inhibitors may disrupt the regulation and enhance the LMP1 and LMP2A mediated activation of Wnt/beta-catenin pathway. In NPC, these viral latent proteins may induce beta-catenin activity through activation of PI3K/AKT pathways.[119,120,122] Activated AKT, phosphorylated GSK-3 and nuclear beta-catenin accumulation were found in NPC.[131] Interestingly, *PIK3CA*, a gene coding for the catalytic subunit p110alpha of PI3K, is amplified and may also cause deregulation of AKT pathway in a subset of NPCs. The findings support that both EBV infection and genetic/epigenetic changes contribute to the constitutively activation of PI3K/AKT and WNT/beta-catenin signaling in NPC. Thus, further comprehensive elucidation of both EBV-related and EBV-independent mechanisms involved in deregulation of critical signal transduction pathways (e.g., STAT, MARK, NOTCH and TGFbeta) from representative EBV-positive NPC tumor lines and primary tumors is important for deciphering the molecular basis of this cancer.

MOLECULAR GENETIC CHANGES IN PRE-INVASIVE LESIONS

Despite of the high frequency of NPC, pre-invasive lesions of nasopharynx were encountered only rarely (~0.6%) during routine examination of nasopharyngeal biopsies in endemic area. To date, very little is known about the molecular changes in these pre-invasive lesions. Activation of telomerase and overexpression of BCL2 were consistently found in the dysplastic lesions.[132-134] These events may contribute to maintaining telomere length and enhancing survival of the pre-invasive epithelial cells. Earlier report has demonstrated clonal EBV genome and latent transcripts including LMP1 in high grade dysplasia and carcinoma in situ.[5] We have also detected EBV latent infection in the high grade dysplasia, but not in the low grade dysplasia and normal nasopharyngeal epithelia.[16] However, in vitro study has proven that EBV infection alone is not sufficient to transform immortalized nasopharyngeal epithelial cells although the viral latent products can induce the invasive property and modulate multiple signaling cascades.[19] Accumulation of other genetic changes might be necessary for malignant transformation of the EBV-infected cells. LOH analysis of microdissected nasopharyngeal epithelia has revealed high frequencies of chromosomes 3p and 9p deletions in the dysplastic lesions and histologically normal epithelia.[16,17] Interestingly, allelic loss at 3p and 9p in the normal nasopharyngeal epithelia is significantly higher in the population from endemic area than non-endemic region. These findings suggested the field cancerization may be common in nasopharyngeal epithelia of Southern Chinese. The occurrence of multiple genetic instable lesions in this population may associate with the exposure to specific environmental carcinogens that increase the susceptibility to further genetic damages. The specific clonal genetic changes disrupting cellular mechanisms (e.g., cell cycle regulation, genetic stability) and signaling pathways (e.g., NF-κB pathway) may predispose for EBV infection, maintenance of permanent viral latency, and tumor initiation. Our recent work has shown the aberrant methylation of *RASSF1A*, the critical tumor suppressor on 3p21.3, in pre-invasive lesions. In this study, multiple dysplasia lesions of the nasopharynx from two Chinese patients were microdissected for investigation.[8,10] *RASSF1A* methylation was detected in some of the microdissected nasopharyngeal epithelia, either with or without EBV infection (Fig. 3). Thus, inactivation of *RASSF1A* may be already involved in the early development of NPC. Interestingly, homozygous deletion of *INK4/ARF* locus, downregulation of *RASSF1A* and activation of NF-κB pathway were also noted in the telomerase-immortalized nasopharyngeal epithelial cells. In vitro selection of the clonal immortalized cells with *INK4/ARF* and *RASSF1A* abnormalities suggests that those changes may be important for in vivo formation of immortalized nasopharyngeal cells.

TUMORIGENESIS MODEL OF NPC

Although there is limited information on pre-invasive lesions, studies suggested that genetic and epigenetic changes collaborate with EBV latent infection in disrupting major cellular mechanisms that contribute to the initiation and progression of NPC. Based on these exciting findings, a collaborative model for NPC tumorigenesis driven by specific genetic and environmental factors is proposed. In individuals from endemic regions, the NPC-associated genotypes for various alleles (such as HLA and the polymorphic

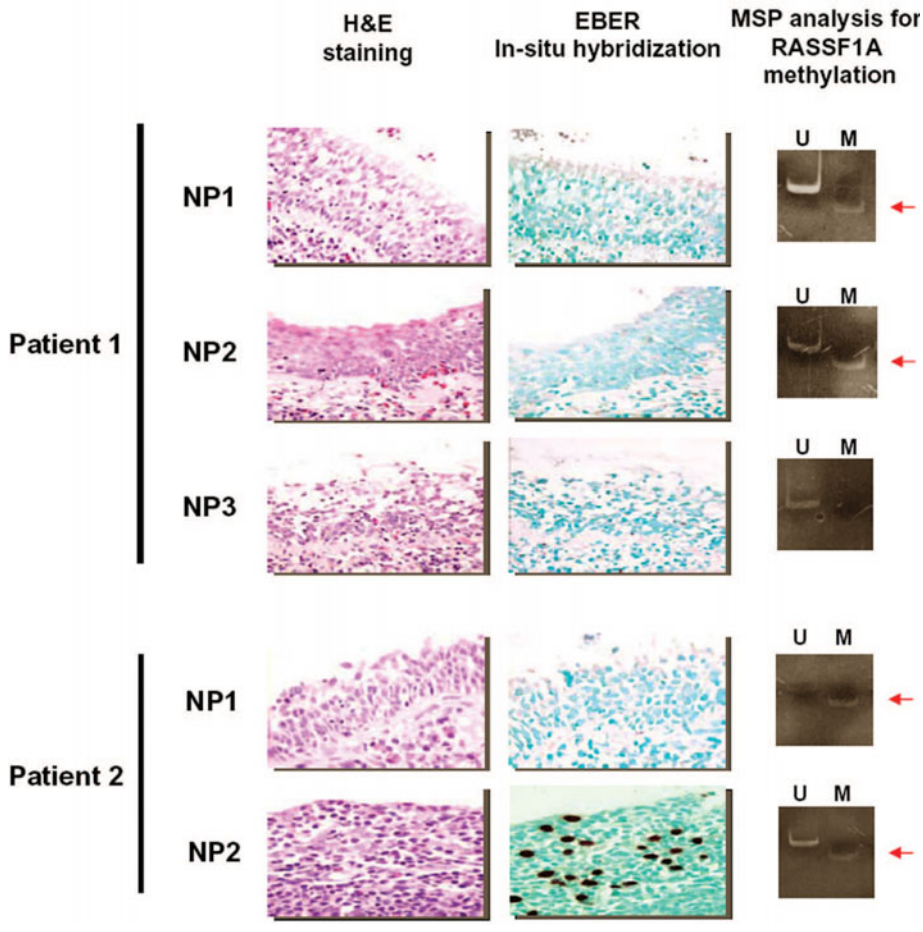

Figure 3. RASSF1A methylation in the precancerous lesions of nasopharynx. The methylation status of RASSF1A promoter in nasopharyngeal dysplastic lesions from five Chinese patients were examined by methylation specific PCR (MSP) analysis. Aberrant methylation (red arrows) was detected in multiple microdissected dysplasic lesions in 2/5 cases (NP1-2 of Patient 1 and NP1-2 of Patient 2). In-situ hybridization for EBER shows that only one lesion (NP2 of Patient 2) is positive for EBV latent infection. H & E: Haematoxylin and Eosin staining; U: unmethylated allele; M: Methylated allele.

genes for carcinogen metabolism, detoxification and DNA repair) may predispose the nasopharyngeal epithelial cells to DNA damage. As a consequence of chronic exposure to specific carcinogens (e.g., nitrosomine), increased DNA damage may lead to the formation of multiple lesions with clonal genetic changes in nasopharynx. The high frequencies of 3p and 9p loss in these lesions are likely due to the growth advantage achieved by p14, p16 and RASSF1A repression. Inactivation of Rb and p53 pathways through loss of *INK4/ARF* locus is critical for immortalization and resistance to

apoptosis of these clones. Suppression of *RASSF1A* in nasopharyngeal epithelial cells may inhibit differentiation and induce genetic instability. Phenotypic and morphological changes occur in these low-grade dysplastic lesions. Chronic inflammation induced by virus infection and chemical carcinogens may lead to persistent NF-κB activation and predispose the pre-invasive lesions to EBV latent infection. The virus may infect these epithelial cells through cell-cell contact with the EBV-bearing B lymphocytes or through polymeric IgA medicated mechanism. EBV latency is stably maintained in one of these progenitor cells. The latent viral gene products will drive the progenitor cell to rapid clonal expansion and invasion. EBV latent proteins, such as LMP1 and LMP2A, may modulate multiple signaling cascades, enhance genetic instability and induce epigenetic alterations. Through activating DNA methyltransferase and polycomb proteins by EBV oncoprotein LMP1, a number of NPC-associated genes will be transcriptional silenced by promoter methylation during the tumor initiation. Multiple cellular mechanisms (e.g., cell proliferation, apoptosis, genomic stability and cell adhesion) and signaling pathways (e.g., NF-κB, AKT, Wnt pathways), including those originally modulated by LMP1, may be permanently disrupted by both epigenetic and genetic changes under the continual selection process. LMP1 expression is then downregulated in a majority of invasive tumors to avoid its cytotoxic effect on epithelial cells. Furthermore, the genetic alterations on 11q, 13q, 14q, 16q may be involved in later steps during development of NPC. Inactivation of *TSLC1*, *THY1*, and other unknown genes may contribute to the late progression and metastasis of NPC (Fig. 4).

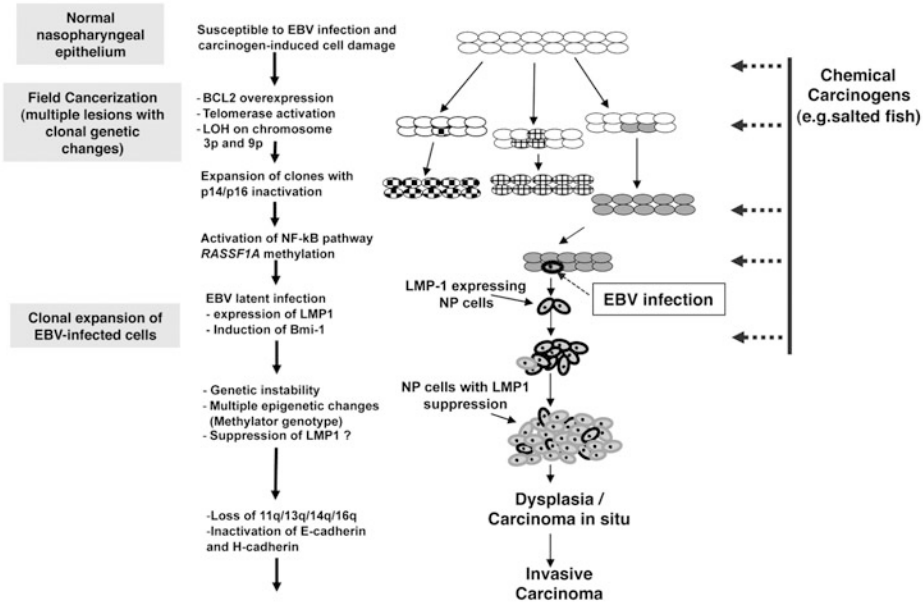

Figure 4. Tumorigenesis model for EBV-associated nasopharyngeal carcinoma.

CONCLUSION AND FUTURE DIRECTIONS

NPC represents an exciting model for the understanding of complex interactions among genetic, environmental and viral factors in human tumorigenesis. The availability of only small biopsies and the rich infiltrate of lymphocytes and plasma cells among cancer cells remain as the challenges of NPC research. Since NPC is strongly associated with EBV, one should be cautious in interpreting and extrapolating the laboratory findings of EBV negative cell line models. However, limited numbers of EBV positive NPC lines are established so far. Studies in the NPC pre-invasive lesion would certainly enhance our insights in the early genetic and epigenetic events and interactions with EBV infection. Nevertheless, investigations are limited by the rarity of pre-invasive lesions encountered in routine biopsies. The knowledge gap may be partially overcome by the establishment and in-depth studies of immortalized nasopharyngeal epithelial cell lines.

Knowledge of critical genetic and epigenetic events in NPC has been rapidly accumulated in the past two decades. Although our insight of this fascinating cancer is greatly enhanced, there are areas still require for more in-depth and active research. Genetic loses and amplification region in NPC genome have been delineated. However, potential important tumor suppressor genes and oncogenes located in some of theses region are not yet discovered. Recently, the next generation massive sequencing technology is rapidly transforming basic cancer biology and biomedicine by decoding DNA sequence of entire cancer genome. We believe that these advanced massive parallel genome sequencing approaches will help us to unveil the unknown driver events for NPC development via establishment of comprehensive catalogs of somatic alterations from NPC genome. Methylation of multiple cancer related genes is common in NPC. Although expression of viral oncoprotein, like LMP1, is implicated, the precise mechanism of this wide-spread methylation is unclear. Further studies are needed to dissect the complex interactions among the various down-stream targets and signaling pathways altered by both host and EBV gene expression. In particular, genetic or epigenetic changes leading to alterations in the inflammatory and immune responses are the exciting field to explore.

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CHAPTER 6

CELLULAR INTERACTIONS IN NASOPHARYNGEAL CARCINOMAS

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Abstract: Tumor cell population in nasopharyngeal carcinomas (NPC) is highly heterogeneous. In addition of being heavily infiltrated by nonmalignant leucocytes, malignant NPC cells can display various phenotypes in terms of epithelial maturation and viral gene expression. These various cell sub-populations communicate through membrane contacts, secretion of cytokines and exosomes. Understanding their interactions is crucial for the elucidation of tumor growth and immune escape as well as for designing better therapeutic approaches. This chapter deals with three major questions. (1) What are the local factors responsible for leucocyte attraction and retention in NPC tumors? (2) What are the suspected autocrine and paracrine mechanisms of tumor growth? (3) What are the mechanisms of tumor immune evasion which could explain the growth of malignant epithelial cells containing viral antigenic proteins in a context of local inflammation?

INTRODUCTION

One of the most striking and consistent characteristic of NPC is the presence of a very abundant leucocyte infiltrate containing mainly T-lymphocytes. This infiltrate often accounts for a large fraction of the tumor mass. Therefore, among the pathologists who originally described NPC, several authors called this tumor lympho-epithelioma (see Chapter 2 by Nicholls and Niedobitek).[1,2] This designation suggested a dual phenotype of NPC cells, in other words, the combination in the same tumor of malignant lymphoid and epithelial cells. This hypothesis has been ruled out in the late years 1970.[3] It is now

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obvious that only epithelial cells are malignant and latently infected by EBV. When NPC are successfully xenografted on nude mice, the leucocyte infiltrate is rapidly eliminated.[3,4] Moreover, although the leucocyte infiltrate is very consistent in the primary tumor, it is generally absent in visceral metastatic lesions (see Chapter 2 by Nicholls and Niedobitek).[1,5] However, there are clinical and experimental observations suggesting that the lymphoid infiltrate plays a role in tumor growth at least at the initial stage of tumor development. This chapter will address several issues related to the formation and function of this infiltrate: what are the inflammatory cytokines produced by malignant NPC cells? Which vascular factor could favor leucocyte entry in the tumor? Conversely, which factor from the leucocyte infiltrate are likely to influence NPC cell growth and survival? We will also comment the role of other potential players involved in NPC tumor growth; for example stromal fibroblasts or rare malignant epithelial cells entering the lytic cycle. Finally this chapter will address a major paradox of NPC physiopathology which is the failure of the immune system to prevent tumor growth despite the presence of antigenic viral products in malignant cells and the presence of multiple immune effectors in the tumor tissue.

OUR LIMITED KNOWLEDGE OF NPC HISTOGENESIS. THE HYPOTHESIS OF A TUBAL TONSIL EPITHELIAL ORIGIN

Primary lympho-epithelial carcinomas with an histological appearance almost identical to NPC have been reported in various anatomic sites outside the nasopharynx, for example in the stomach and salivary glands (consistently associated to EBV), in the lung (consistently associated with EBV in Asian population) and thymus (rarely associated with EBV).[6-10] However one should acknowledge that these "ectopic" primary NPCs are extremely rare. A contrario, it should be recognized that the overwhelming majority of EBV-associated lymphoepithelial carcinomas do occur in the nasopharynx. This observation suggests that NPC oncogenesis is strongly dependent on local factors related to anatomical, histological and physiological characteristics of the nasopharyngeal cavity. Such local characteristics are probably important not only for EBV-infection of epithelial cells but also for the formation of the leucocyte infiltrate. On the basis of direct or endoscopic observation of small tumors, it is well established that NPC consistently arise in the fossa of Rosenmuller which are lateral extensions of the nasopharyngeal cavity also called "pharyngeal recesses".[11] These recesses are situated just above the Eustachian tube openings. They are also in close proximity to the "tubal tonsils" which is a part of the Waldeyer ring. This ring of lymphoid structures comprises the nasopharyngeal tonsil (NT) or adenoid, attached at the roof of the pharynx; the paired tubal tonsils (TT) as mentioned previously; the paired palatine tonsils (PT) positioned in the oropharynx; and the lingual tonsil (LT) on the posterior third of the tongue.[12] At least some of these human lymphoepithelial elements are homologue to a unique rodent lymphoid structure called nasopharynx-associated lymphoid tissue (NALT). In rodents, NALT is located on both sides of the nasopharyngeal duct which is homologous to the human nasopharyngeal cavity.[13]

There are specific characteristics applying to each elements of the Waldeyer ring. PT and LT are directly exposed to ingested pathogens and antigens whereas NT and TT are strategic for interactions with airborne pathogens and antigens. The surface of the nasopharyngeal tonsils (NT and TT) are covered mainly with a ciliated respiratory epithelium whereas that of oropharyngeal tonsils (PT and LT) are protected by stratified squamous nonkeratinized or parakeratinized epithelium respectively. Unfortunately from

the point of view of NPC biology, among the elements of the Waldeyer ring, TT have not been in the focus of most biological investigations. Very few things are known about tubal tonsils (TT). However we know that all human tonsils share common structural and functional properties. The lymphoid subepithelial compartment contains germinal centers surrounded by a mantle zone which are more or less similar to their lymph node homologs. Two major epithelial components are the surface epithelium and the epithelium lining the crypts which has the structure of a sponge with interstices containing infiltrating T and B cells, macrophages, interdigitating dendritic cells and Langherans cells.[12] This specialised reticulated crypt epithelium, often called lymphoepithelium, has the same embryonic origin as the thymic epithelium (third pharyngeal pouche) and expression of a distinct subset of cytokeratins (CK 8, 18 and 19).[14] The tonsil crypt epithelium has been sometimes described as a model of "lymphoepithelial symbiosis". In addition, tonsil lymphoepithelium has a network of intra-epithelial capillary vessels, some of them ending in high endothelial venules (HEVs) with specific expression of adhesion molecules favoring lymphocyte extravasation.[12]

Because tonsillar epithelium has specific features supporting lymphoepithelial interactions, there is a suggestion that NPC cells derive from tubal tonsillar epithelial cells. This hypothesis needs to be substantiated by more experimental evidence. We know that small numbers of epithelial cells from both tonsil surface and reticulated crypts can be infected by EBV in vitro but we do not know yet whether specific markers of the tonsil crypt epithelium are expressed by NPC cells.[15] We also ignore whether NPC tumor vessels have some properties in common with tonsillar lymphoepithelium vessels that would favor lymphocytes infiltration. Even if such vessels are not present in the tumor, their presence in close proximity to the tumor in tonsil crypts and interfollicular spaces might facilitate leucocyte entry in the neighbouring tumor tissue. Answering these question will be essential to better understand the formation of the lymphoepithelial stroma and its possible influence on tumor growth.

SUBPOPULATIONS OF STROMAL CELLS IN NPC TUMORS

Infiltrating Leucocytes

As previously mentioned, the abundance of infiltrating leucocytes is a major characteristic of NPC tumor stroma (in many reports, the terms "tumor infiltrating lymphocytes" or TILs are synonymous of "tumor infiltrating leucocytes"). Infiltrating leucocytes are often located around malignant cell clusters but sometimes disseminated within epithelial cell nests (see Chapter 2 by Nicholls and Niedobitek).[1] As shown by immunohistochemistry using an anti-CD3 antibody, most of them are CD3-positive T cells with a morphology of small resting lymphocytes.[16] Among the CD3-positive cells, CD8 and CD4 T cells are present in varying proportions depending on the tumor specimens.[17] Collagenase dispersion of cells from tumor pieces allow quantitative assessment of subpopulations of tumor infiltrating leucocytes by flow cytometry. Using this technique, Ferradini et al have found a CD4 to CD8 ratio varying from 0.4 to 2.2.[18] On average, 15% of these T cells express the integrin $\alpha E\beta 7$ (or CD103), a surface marker frequently expressed by intra-epithelial lymphocytes. NK-cells positive for CD56 or CD94 are also detected both by flow cytometry on TILs (about 5% TIL) or immunohistochemistry.[18,19] Small populations of B cells stained with anti-CD19 or -CD20 are also consistently

detected.[17,18] Several studies have reported the presence of monocytes and dendritic cells in NPC biopsies.[20-23] Dendritic cells are often found inside malignant cell nests whereas monocytes are more often interspersed at some distance of epithelial cell clusters.[21] Some NPC dendritic cells occasionally display CD23 expression like follicular dendritic cells.[24] A fraction of dendritic cells contained in NPC tumors have features of Langherans cells (also called T-zone histiocytes) (see Chapter 2 by Nicholls and Niedobitek).[1,22] Eosinophils are also detected in the leucocyte infiltrate of NPC tumors.[25,26]

Recently, several studies have refined the description of infiltrating T-lymphocytes contained in NPCs (Fig. 1). Lau et al have shown that about 12% of TILs recovered after collagenase cell dispersion have a phenoptype of regulatory T cells (T-reg), $CD4^{+}CD25^{high}$, most of them being Foxp3-positive and CCR7-negative.[27] By immunohistology on tissue sections, it has been shown that CD25 high and Foxp3 T cells are more abundant in tumor tissue than in nonmalignant nasopharyngeal mucosa.[28] On the other hand, lymphocytes expressing CXCR3 (the CXCL10 or IP10 receptor) are consistently detected in NPC tissue sections; in contrast, they were apparently rare in squamous carcinomas of the tongue investigated in the same study.[29] CXCR3 expression is generally associated with Th1 differentiation. Detection of a subpopulation of CXCR3-positive TILs suggests that Th1 differentiation is taking place inside NPC tumors despite the presence of regulatory T

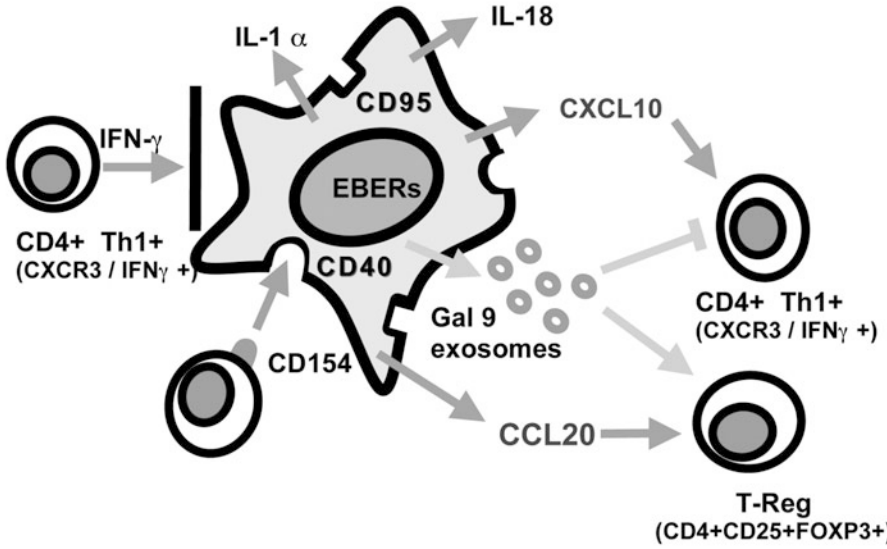

Figure 1. Summary of lympho-epithelial interactions in NPC tumors. Malignant epithelial cells are admixed with several sub-populations of T-lymphocytes. CD4+ Th1 T cells are characterized by surface expression of CXCR3 and production of interferon γ (IFNγ). Regulatory T cells are characterized by co-expression of CD4, CD25 and intra-cellular Fox P3. Malignant NPC cells produce various interleukins (IL-1 and -18) and chemokines, mostly with inflammatory effects. CXCL10 (or IP10) is suspected to enhance expansion of CD4+Th1 cells.[29] CCL20 is probably involved in the expansion of regulatory T cells (T-reg).[53] Malignant NPC cells also release exosomes containing galectin 9 which are expected to enhance T-reg expansion and to induce apoptosis of mature CD4+ Th1 cells expressing Tim-3.[89] A large fraction of infiltrating T-lymphocytes express CD154, the CD40-ligand. According to in vitro experiments, CD154 provides survival signals to malignant epithelial cells, with effects antagonist of CD95 stimulation.[57] Resistance of malignant NPC cells to interferon γ is thought to be supported by small noncoding EBV RNAs called EBERs.[56]

cells. CXCR3-postive cells are often detected within nests of malignant cells.[29] It will be interesting to know whether intra-tumoral T-reg have the same distribution or accumulate at some distance of the tumor cells.

Nonleucocyte Stromal Cells

So far, only limited attention has been paid to nonleucocytic stromal cells in NPCs, especially fibroblasts and endothelial cells although both cell types are present in the tumor tissue. Histological forms of NPC with large areas of fibroblast proliferation have been reported.[30] Unfortunately, so far, there has been no biological characterization of the fibroblasts derived from NPC tumors as well as from the normal nasopharyngeal mucosa (although it is much easier to recover stromal fibroblasts than malignant NPC cells when growing tumor cells in vitro; P. Busson, personal observations). The potential growth promoting effect of stromal fibroblasts on malignant NPC cells remains almost completely unexplored. In experimental models of breast carcinomas, CXCL12 or SDF1 (stromal cell-derived factor 1) has proven to be an important mediator of the growth-promoting effect of stromal fibroblasts on carcinomas cells.[31] According to a recent study, malignant NPC cells often strongly express CXCR4 which is the physiological receptor of CXCL12.[32] However, CXCR4 has a nuclear localisation in malignant cells of most NPC biopsies.[32] A distribution which might not be compatible with responsiveness to CXCL12. In addition, production of CXCL12 by NPC stromal fibroblasts remains to be investigated. As reported for other tumors, VEGF is often detected by immunohistochemistry in nasopharyngeal carcinomas. Its level seems to be related to tumor aggressiveness.[33]

HETEROGENEITY OF MALIGNANT CELLS IN NPC TUMORS

In addition to being heavily infiltrated by various categories of nonmalignant cells, the tumor cell population itself is heterogeneous in NPC tumors. This heterogeneity is obvious in terms of epithelial differentiation. Although most NPCs are nonkeratinizing undifferentiated carcinomas, one can consistently find areas of squamous cell maturation in this type of tumors (see Chapter 2 by Nicholls and Niedobitek).[1] One can assume that there is an inverse relationship between proliferation and differentiation, although this has not been formally proven for NPC cells. It is not yet known whether one can isolate tumor stem cells from NPC specimens. A recent publication by Wang et al has reported the presence of stem cell-like side population cells in the CNE2 cell line which was depicted as an NPC cell line.[34] This “side-population” or “SP” phenotype which is demonstrated by flow cytometry rely on a capacity of active extrusion of the DNA binding dye Hoechst 33342. It is characteristic of stem cells in several normal tissue lineages as well as in some malignant tumor lines. Wang et al have found that a fraction of 2.6% CNE2 cells display an SP phenotype which is associated with a series of characteristics suggestive of stem cell behavior. By comparison with nonSP cells, CNE2 SP cells have a much higher clonogenic potential in vitro. They are more tumorigenic in SCID mice and more resistant to some cytotoxic drugs. They also have a higher production of interleukin 19. One limitation of this study is that CNE2 cells are not representative of NPC cells. For example, they are not latently EBV-infected. Nevertheless, it is obvious that characterization of NPC tumor stem cells will become a major field of investigation in the next years. It will be important to determine which factors control NPC stem cell

asymmetrical division and transient proliferation of tumor cells that leaves the stem cell compartment. It will be also important to confirm that morphological epithelial maturation is associated with both loss of stemness and decrease of proliferation.

Qualitative and quantitative variations in EBV-gene expression is another major factor of heterogeneity among NPC tumor cells. Another chapter of this book (Chapter 4 by Gourzones et al) is focused on EBV gene expression and their role in tumor development. In the scope of this chapter we will simply mention a few points in close connection with tumor heterogeneity and cellular interactions. There is a consensus that EBV-infection is mainly latent in NPC. However, the amount of EBV latent gene products is variable from one cell to another, especially the amount of the Latent Membrane Protein 1 (LMP1).[35] EBV viral particles have never been observed in fresh NPC biopsies although they can be produced by NPC cells used in short term culture in vitro and incubated with BUdR or other inducers of viral replication.[36-38] Nevertheless partial expression of EBV genes involved in the lytic-productive cycle can occur in NPC tumors in situ. Several EBV-proteins specifically expressed during the lytic cycle have been detected in NPC tissue sections in limited areas of the tumor; for instance, elements of the EA complex, the BZLF1 protein and the EBV-Dnase.[39-41] Consistently, linear replicative forms of the EBV genome are occasionally detected in a subset of NPC biopsies.[42] The factors which control partial expression of the lytic EBV-genes in NPC cells are still poorly understood. There is some evidence that maturation from a phenotype of immature basal cell toward a phenotype of intermediate squamous tumor cells favors lytic gene expression.[43] Local production of TGF β is also suspected to increase lytic cycle gene expression in some malignant epithelial cells.[44] Recent findings made on EBV-associated SCID lymphoma models has spurred a renewed interested for lytic cycle gene expression in NPC. Hong et al have shown that a minority of lymphoma cells entering the lytic cycle play a critical role in the emergence of the disease through production of cytokines and angiogenic factors, especially interleukin-6.[45] Similar mechanisms might be important for NPC tumor growth although interleukin-6 does not seem to be very abundant in NPC tissue sections.[46]

DYNAMIC CELLULAR INTERACTIONS: POSSIBLE CONTRIBUTION TO TUMOR GROWTH

Role of Cytokines in Leucocyte Attraction and Retention

The leucocyte infiltrate consistently account for about 50% of the tumor mass. Obviously, it cannot be accounted for by a remnant of tonsilar leucocytes pre-existing to tumor development. For this reason, we hypothesized a long time ago that malignant epithelial cells were playing an active role in the formation of the infiltrate (Fig. 1, Table 1). Initially, we could demonstrate that malignant NPC cells constitutively produce interleukin-1 alpha (IL-1α), a cytokine with various inflammatory effects, including T-cell proliferation.[47] This observation was later confirmed by Huang et al (1999) who detected both IL-1 α and β in malignant NPC cells by antibody staining of tissue sections. Simultaneously, investigations made by RT-PCR demonstrated IL-1 α and β transcripts in most NPC primary tumors and a fraction of metastatic lesions and its absence in control fragments of nonmalignant nasopharyngeal mucosa.[48] Production of other inflammatory cytokines was investigated in NPC specimens in the subsequent years. IL-18 which has structural similarities with IL-1 is known to stimulate the proliferation of activated T cells,

Table 1. Main cytokines involved in NPC cell interactions*

Designation	Synonyms	Biological Properties and Relevance to NPC (references in the chapter)
CCL2	MCP1	Agonist of the CCR2 receptor. Chemoattracts monocytes, memory T cells, NK cells and immature dendritic cells. Produced by CD68+ monocytes in NPC tumors (ref. 23).
CCL3	MIP1-α	Agonist of the CCR5 receptor. Recruits CD8+ T cells. Produced by CD68+ monocytes in NPC tumors (ref. 23).
CCL20	MIP3-α	Chemoattracts lymphocytes and dendritic cells. Signals through the CCR 6 receptor. Produced by malignant NPC cells (ref. 53).
CXCL10	IP10	Induces chemotaxis of activated T cells Agonist of the CXCR3 receptor which is expressed by a fraction of Th1+ T cells in NPC tumors. Produced by malignant cells (ref 29).
CXCL12	SDF-1 (stromal cell-derived factor 1)	Agonist of the CXCR4 receptor. Its role in NPC remains to be clarified (refs. 31 and 32).
Hepatocyte Growth Factor	HGF—Scatter factor	Agonist of Met. Broad effects on cell proliferation and tissue morphogenesis Produced by stromal cells in NPCs whereas Met is expressed by malignant cells (ref. 60).
Interferon-γ	IFN-γ	Regulates the antigen-specific phase of the immune response. Abundantly produced by CD3+ T cells and CD94+ NK cells within the NPC tumor infiltrate (refs. 23 and 54).
Interleukin-lα	Hemopoïetin 1	Pro-inflammatory. Cell-associated. Produced by malignant NPC cells (refs. 47 and 48)
lnterleukin-1β	LAF (lymphocyte-activating factor)	Pro-inflammatory. Secreted in the extra-cellular medium. IL-1α and β have similar biological properties. Produced by malignant NPC cells (ref. 48).
lnterleukin-10	IL-10	Inhibits expression of pro-inflammatory cytokines like IL-1 and TNF. Promotes Th2 differenciation. Its presence in NPC tumors is a matter of controversy (refs. 46, 49, 50 and 51).
lnterleukin-18	Interferon-γ inducing factor	Enhances production of γ-interferon by activated T cells and promote Th1 differenciation. Produced by malignant NPC cells (ref. 19).
Stem cell factor	SCF-c-kit ligand	Involved in cell proliferation and differentiation especially in hematopoiesis and melanogenesis. Co-expressed with its receptor c-kit by malignant cells in most NPC tumors. Autocrine effects not proven (refs. 62 and 63).

*Chemokines which are identified by the suffix CC or CX are small cytokines with chemotactic activity. CC chemokines have two adjacent cysteins near their N-terminus whereas in CX chemokines the two N-terminal cysteins are separated by one amino-acid.

to enhance their production of γ interferon and to promote their Th1 differentiation. By immunohistochemistry, IL18 was shown to be consistently produced by malignant NPC cells but not by epithelial cells of the non malignant mucosa.[19] Data regarding IL-10 in NPCs remain controversial. According to three publications based on immunohistochemistry, IL-10 is detected in malignant cells of about 60% primary tumor biopsies of NPCs.[49-51] In contrast, Beck et al (2001) has failed to detect IL-10 transcripts by in situ hybridization in malignant NPC cells whereas in some cases it was detected in the leucocyte infiltrate. The same group has also found a very rare expression of IL-6 and IL-8 transcripts by malignant NPC cells contrasting with occasional expression by infiltrating leucocytes.[46]

More recently, several studies have been focused on CC chemokines. Using in situ hybridisation, Teichman et al demonstrated a consistent and intense expression of the CXCL10 cytokine messenger by malignant NPC cells (CXCL10 is also called IP10 for γ-interferon inducible protein 10).[29] CXCL10 induces chemotaxis of activated T cells and inhibits angiogenesis. It is the agonist of the CXCR3 receptor.

As mentioned previously, CXCR3 which is often associated with Th1-differentiation is consistently detected in a fraction of T cells infiltrating NPC. However, there is no precise relationships between CXCL10 and CXCR3 expression in terms of abundance or spatial distribution in the tissue sections.[29] To our knowledge CXCL10 production has not yet been confirmed at the protein level in NPC tissue sections. Its status in the nonmalignant nasopharyngeal mucosa is not known. CCL20 or MIP-3α (Macrophage inflammatory protein-3 α), is another CC-chemokine that induces leukocyte migration into inflammation sites and regulates leukocyte trafficking through lymphoid tissues. It is a chemoattractant for memory regulatory T cells.[52] Chang et al have reported a high expression of CCL20 in NPC tumor cells. Interestingly, CCL20 is detected at a high concentration in serum samples from NPC patients. Its concentration is correlated with tumor mass and has prognostic value.[53]

In summary, on the basis of currently published data, the main inflammatory cytokines produced by malignant NPC cells are IL-1 α and β, CCL20, IL-18 and probably CXCL10 (Fig. 1, Table 2). We do not know yet which factors up-regulate their production by NPC cells. So far there is no evidence of a direct role of an EBV product in their induction. Except for CCL20 whose expression is induced by EBNA1 in the background of malignant Hodgkin cells.[52] Whether or not the same applies to epithelial cells remain to be investigated. All these cytokines are produced by NPC cells not only in situ but also by several NPC tumor lines used in the laboratory, suggesting that their production is constitutive and do not require the presence of the leucocyte infiltrate.[19,29,47] However some leucocytes might be involved in positive regulatory loops contributing to additional infiltration. For example CD3-positive T-lymphocytes and CD94 NK cells abundantly produce γ-interferon when they are located in primary NPC tumors whereas they do not or at a very low level when they are in nonmalignant NP mucosa.[23,54] Concentration of γ-interferon is increased in the plasma of NPC patients.[55] Production of γ-interferon by infiltrating lymphocytes is thought to be induced by local IL-18 whereas γ-interferon will in turn enhance CXCL10 production by malignant epithelial cells.[19,29] Similarly, CD68-positive monocytes have been shown to abundantly produce two chemokines, CCL2 (also called MCP1 or monocyte chemoattractant protein 1) and CCL3 (also called MIP-1α), when they are located in the tumor infiltrate but not—or at a low level—when they are observed in nonmalignant nasopharyngeal mucosa or sub-mucosa.[23] CCL2, like other monocyte chemoattractant proteins, recruits

Table 2. Peripheral blood modifications related to cell interactions in NPC tumors

Parameter	Change in NPC Patients	Clinical Relevance	References in the Chapter
Plasma soluble CD 23	Increase	Independent pronostic factor for locally advanced nonmetastatic NPCs	92
Plasma soluble CD40-L	Increase	Evidence of a correlation with CD40-L expression by tumor infiltrating lymphocytes	93
Plasma CCL20	Increase	Initial level above 65 ng/ml predictive of an increase in the risk of recurrence	53
Plasma interferon-γ	Increase	Not Determined	55
Plasma TGF-β	Increase	Higher levels in patients with advanced stages of the disease	78
Overall CD4 cell count	Decrease	Not Determined	27
CD4+CD25+FOX P3 cell count	Increase	Not Determined	27,55
Membrane expression of CD40-L by stimulated CD3+CD8−	Major decrease	Not Determined	93

and/or activates monocytes, activated T cells, NK cells and immature dendritic cells.[23] CCL3 is an attractant for CD8+ T cells, B cells and dendritic cells.

Tumor infiltrating leucocytes which are so abundant in the primary tumor often disappear in the metastatic lesions.[5] This might be explained by the decrease or loss of the production of some cytokines during the metastatic process. Alternatively, a positive balance between leucocyte entry and exit in the primary tumor might be dependent on specific anatomic factors in its local environment. As mentioned earlier, the proximity of the tubal tonsils with their network of specialised vessels—especially "high endothelial veinules"—probably facilitates leucocyte extravasation and tissue penetration.[12]

Influence of the Leucocyte Infiltrate on Malignant NPC Cells

It is obvious that malignant NPC cells are resistant to growth-inhibitory factors released by the leucocyte infiltrate, especially γ-interferon. Resistance to interferon is thought to be supported by at least 1 type of EBV-products, the small untranslated RNAs called EBERs (small EBV-encoded RNAs). EBERs 1 and 2 are about 120 base long and are very abundant in NPC cells. They have several oncogenic functions. One of these functions is to prevent the blockade of protein synthesis induced by interferons; an effect which is dependent on their direct binding to PKR.[56]

Not only the leucocyte infiltrate fails to block NPC tumor growth but it is suspected to enhance malignant NPC cell growth at the initial stage of primary tumor development.

This hypothesis of a « ping-pong » or « folie à deux » mechanism is based on two main observations. One is the consistency of the infiltrate present in virtually all NPC primary tumors. The other is the low rate of successful NPC xenografts when using fragments from primary tumors (about 1% successful grafts) in contrast to a rate of about 50% when using metastatic fragments.[4] The same contrast has been reported for other types of human tumors but probably not to the same extent. One possible interpretation is that infiltrating cells which are not retained in nude or SCID mice are required for growth of cells from primary tumors whereas cells from metastatic lesions are much better prepared for autonomous growth.

One experimental argument in favour of a cooperative effect of infiltrating leucocytes in tumor growth is provided by data from our and other groups who have investigated the role of the CD154/CD40 system in NPC cell survival and growth. Indeed CD40 is consistently and abundantly expressed by NPC cells whereas its cognate ligand CD154 or CD40-L is consistently expressed by infiltrating T cells.[17] We have shown that the CD40-receptor is functional in NPC cells in at least one respect; it has the ability to induce a cellular response called "rapid rescue from CD95-induced apoptosis".[57,58] In other models, long term permanent stimulation of CD40 has been shown to enhance the tumorigenic phenotype of epithelial cells.[59] In summary, high constitutive expression of CD40 by malignant NPC cells and infiltration by T cells bearing CD154 is likely to favour oncogenesis and tumor development. CD70 (strongly expressed by NPC cells) and CD27 (expressed by many infiltrating lymphocytes) are suspected to be involved in a similar process; a point that would require further investigation.[17]

In addition, Qian et al have published evidence of lymphoepithelial interactions mediated by the met receptor tyrosine kinase and its ligand the HGF receptor.[60] Met is consistently expressed by NPC cells whereas its ligand HGF is only expressed by stromal cells which have not been unequivocally characterized but are probably infiltrating lymphocytes. A contribution of the Met/HGF system to tumor growth is suggested by an inverse correlation between patient survival and the level of Met expression. In many cell types, Met stimulation is known to enhance cell proliferation and motility.[61] It is not yet known whether pharmacological agents which can block Met phosphorylation and signalling are beneficial to NPC patients.

Other Potential Mechanisms of Growth Based on Cellular Interactions

In addition to lympho-epithelial interactions, other cellular interactions are suspected to contribute to tumor growth. We have previously mentioned an hypothetical role of stromal fibroblasts possibly mediated by the SDF1/CXCR4 ligand-receptor pair. Autrocrine growth mechanisms should also be taken in account (Fig. 2). Sheu et al have suggested a role for an autocrine loop involving the c-kit tyrosine kinase receptor and its ligand SCF (stem cell factor). Both molecules are co-expressed by malignant cells from primary tumors and metastases in about 70% NPC patients. SCF/c-kit co-expression is also observed in epithelial cells of nonmalignant nasopharyngeal mucosa but at a lower frequency.[62] On the other hand NPC patients with c-kit expression in their tumors do not have a more severe prognosis.[63] In our hands, there is no significant constitutive phosphorylation of c-kit in an EBV-positive, c-kit-positive NPC xenograft (P. Busson, unpublished data). As mentioned in Chapter 10 by Hui and Chan, investigations on SU 11248 (sunitinib malate) are still in progress for NPC patients.[64] This drug is active on several tyrosine kinases including c-kit. Positive results would encourage further investigations about the role of

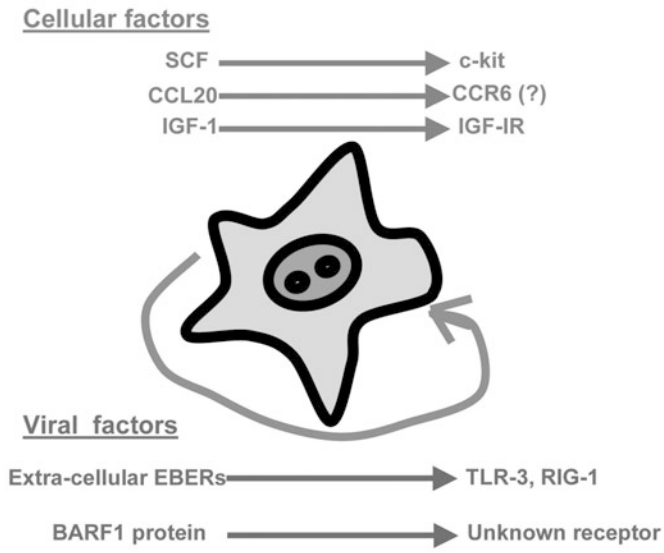

Figure 2. Candidate autocrine growth factors for NPC cells. In a fraction of NPC tumors, there is co-expression of SCF (stem cell factor) and its receptor c-kit with a potential growth-promoting effect.[62,63] CCL20 is a chemokine abundantly produced by NPC cells which is suspected to enhance their migration and invasion (the involvement of the CCR6 receptor has not been yet proven).[53] EBERs are small untranslated viral RNAs which are very abundant in the nuclei of NPC cells. A fraction of them is released in the cytoplasm and the extra-cellular medium where they can activate the TLR-3 and RIG-1 receptors.[68] EBERs induce IGF-1 production in epithelial cells possibly through RIG1 stimulation whereas IGF-1 enhances malignant cell survival and proliferation.[69] A growth promoting effect of the BARF1 protein is also suspected.[66,67]

the c-kit-SCF pair. In addition to promoting leucocyte infiltration, CCL20 or MIP-3α is suspected to have an autocrine enhancing effect on NPC cell migration and invasion.[53]

Several viral products are suspected to participate in autocrine growth mechanisms of NPC cells. A 33 Kd EBV protein called BARF1—according to the name of the corresponding viral ORF—is frequently produced by malignant cells in a large fraction if not all NPC tumors.[65] This protein which is secreted in the extracellular medium has homology with the human CSF gene. According to Houali et al, it is detected in the plasma of NPC patients.[66] A publication from the same group provides evidence that extra-cellular BARF1 has growth-promoting activity and suggest that it might be an autocrine growth factor for malignant NPC cells.[67] The small viral untranslated RNAs called EBERs have been previously mentioned for their role in cell resistance to interferon. These viral RNAs which are partially double-stranded are mainly concentrated in the nucleus. However, a small fraction of them leaks in the cytoplasm and even further in the extra-cellular space. These extra-nuclear EBERs have the power to activate some cellular receptors of double-strand RNAs, such as RIG1 and TLR-3.[68] Activation of these receptors can result in growth-promoting signals for example an increase in IGF-1 secretion.[69] Finally, a possible contribution to tumor growth of a small number of cells entering the lytic cycle has already been mentioned in this chapter.

MECHANISMS OF TUMOR IMMUNE EVASION

Various Mechanisms of Tumor Evasion for EBV-Associated Malignancies

The Epstein-Barr virus is involved in a wide range of human malignancies, either of epithelial or lymphoid origin. In all these malignancies, EBV-infection is mainly latent. No viral particles are detected by electron microscopy in tumor biopsy sections. The EBV genome is under circular form in the nuclei of malignant cells and most viral genes are silent, especially genes encoding viral enzymes and structural proteins. However a few viral genes called latent genes are consistently expressed in EBV-associated malignancies. These genes encode various types of viral products, either proteins or untranslated RNAs. All these viral products are suspected to contribute to the maintenance of the malignant phenotype. Depending on the type of malignancy, three patterns of EBV latency have been identified. The Type III latency is characterized by the expression of a wide range of viral products including the immunodominant EBV nuclear antigens (EBNA) 3-6 and the less immunogenic antigens, EBNA1 and latent membrane proteins (LMP) 1 and 2. This type of latency is observed mainly in lymphoid malignancies occurring in immunocompromised individuals, such as the posttransplant lymphomas. In contrast, the expression of viral products is much more restricted in Type I latency characteristic of EBV-associated Burkitt's lymphomas which do not express LMP1 and LMP2. Halfway between Type I and Type III latency, Type II latency is characterized by the frequent expression of LMP1 and LMP2 combined to the absence of EBNA 3 to 6.[70]

Mechanisms of immune evasion are completely different in these three categories of tumors. In malignancies with Type III latency—mainly posttransplant lymphomas—immune evasion likely occurs due to the direct suppression of lymphocyte function by immunosuppressive drugs.[71] In contrast, malignancies with Type I and Type II latency occur in patients without obvious impairment of immune functions at the systemic level. Tumor immune evasion results from various combinations of 2 types of tolerogenic mechanisms: on one hand, internal cellular alterations impairing the machinery of antigen processing and presentation and, on the other hand, release of factors which create a context of immune inhibition in the tumor microenvironment. Regarding Burkitt's lymphoma (Type I), there is strong evidence that immune evasion is supported to a large extent by early defects of antigen presentation to CD8+ T cells, especially a reduction in MHC class I surface expression.[72] This does not seem to apply to NPC which is characterized by a Type II latency. Defects in MHC class I molecules have been reported in NPC cells, especially in a context of high EBNA1 expression.[73,74] However, malignant NPC cells consistently retain the capacity to process and present antigen to CD8+ T cells.[75,76] Recent observations suggest that several immunosuppressive factors are released by malignant cells in the tumor microenvironment. These immunosuppressive factors probably result in partial inhibition rather than in complete abrogation of the local immune response. One potential consequence of this crippled immune response might be not only a failure in tumor eradication but also the progressive emergence of more resistant malignant clones, contributing to a process of immune selection or immuno-editing.

Candidate Immunosuppressive Factors in NPC Tumor Microenvironment

Factors released by malignant cells can impair local immune reactions by a large variety of mechanisms.[77] Some factors released by tumor cells have direct cytotoxic or inhibitory effects against CD8+ and CD4+ CTL. Other factors have direct promoting effects on regulatory T cells or shift the balance of Th1 and Th2 CD4+ cells towards a predominant Th2 response. Finally other tumor factors can impair the functions of professional antigen presenting cells in a way that results in tolerogenic effects on T-cell distribution and functions.

Only a fraction of immunosuppressive factors reported in various tumor models has been investigated in NPC, including TGF-β, IL-10, Fas-ligand and nitric oxide. We will provide concise informations for each of them. TGF-β can impair anti-tumor immune reactions by its inhibitory effect on CTLs and its stimulating effect on T-reg expansion.[77] An elevated concentration of TGF-β has been reported in the plasma of NPC patients.[78] However, with regard to tumor environment, TGF-β transcripts are not more abundant in the tumor tissue than in the nonmalignant nasopharyngeal mucosa.[48] Fas-ligand can induce apoptosis of actived T cells which often express high amounts of plasma membrane CD95 or Fas-receptor. Production of Fas-ligand by malignant NPC cells has been reported but only in a fraction of patients generally with a high tumor mass.[79] IL-10 is a cytokine which switch the Th1/Th2 balance towards Th2 polarisation. As previously mentioned, its expression in malignant NPC cells remains controversial.[46,49-51] Nitric oxide (NO) is a gas which has strong inhibitory effects on T cells.[80] In humans, NO derives from arginin through the action of enzymes called NO-synthases. Endothelial NO-synthase (e-Nos) has constitutive expression in the vascular system whereas inducible NO-synthase (i-Nos) can be expressed in a wide range of cell types mainly in inflammatory conditions. A high expression of i-Nos has been reported in malignant NPC cells; it is not yet known whether its expression is under the control of latent EBV infection.[81] Nevertheless a high level of NO concentration is probably achieved in NPC tumor environment.

There are a lot of other potential tumor immunosuppressive factors which, to our knowledge, have not yet been investigated in NPC, for example the enzyme indoleamine 2, 3-dioxygenase (IDO) which is constitutively expressed by some human tumors.[77]

Role of Tumor Exosomes

Exosomes are bi-lamellar nanovesicles secreted by many cell types which are paradoxically derived from structures of the endosomal pathway called multivesicular bodies.[82] Exosomes contains various types of cellular proteins which are either luminal or membrane-inserted. They also carry RNAs.[83] There is growing evidence that exosomes are major players in cell communications including developmental processes, neural communications and immune responses.[82,84] Exosomes also appear to play an important role in tumor growth and host-tumor relationships.[85] Initial evidence that exosomes could play a role in immune evasion of EBV-infected cells came from a study by J. Middeldorp's group in Amsterdam dealing with the EBV-encoded LMP1 oncoprotein. They could show that this Type III membrane protein contains an immunosuppressive motif in its first transmembrane segment and is secreted by EBV-transformed B cells in association with exosomes.[86] These LMP1-positive exosomes have an inhibitory effect on T-cell proliferation. More recently, inspired by this study, our group could demonstrate that NPC cells also produce exosomes. These NPC exosomes contain LMP1 only when

they are produced by NPC cells with strong LMP1 expression (for example cells from the C15 xenograft).[87] In contrast, regardless of the presence of LMP1, they have a high content of HLA class II molecules and galectin 9. Galectin-9 is a β-galactosyl binding lectin which is very abundant in NPC and has been identified as one specific ligand of the Tim-3 receptor.[88] Tim-3 is expressed by mature CD4+ Th1 lymphocytes. In the context of CD4+ Th1 lymphocytes, galectin 9 binding to Tim-3 triggers a very rapid process of apoptosis. We have shown that the galectin 9 CRDs (Carbohydrate Recognition Domains) are presented at the surface of NPC exosomes. Accordingly, these exosomes can induce apoptosis of CD4+ T cells expressing Tim-3.[89] In addition, we have demonstrated that HLA-class II-positive exosomes carrying galectin 9 are detected specifically in plasma samples from NPC patients but not from control patients, for example patients with nonNPC head and neck tumors.[89] We believe that this presence of galectin 9 positive exosomes in the plasma reflects passive diffusion from tumor interstitial fluids to the blood stream. In addition to cytotoxic effects against mature CD4+ Th1 lymphocytes, NPC exosomes are suspected to enhance T-reg expansion possibly in cooperation with CCL20 (C. Durieu and P. Busson, personal data).[52] They probably also have some influence on dendritic cell maturation.[90] According to our current hypothesis depicted in Figure 3, NPC exosomes might subvert CD4+ Th1 functions by combining effects on dendritic cells favouring initial Th1 maturation with direct cytotoxic effects on fully mature Tim-3 positive Th1 cells. Development of novel in vitro and in vivo models will be required to validate this hypothesis.

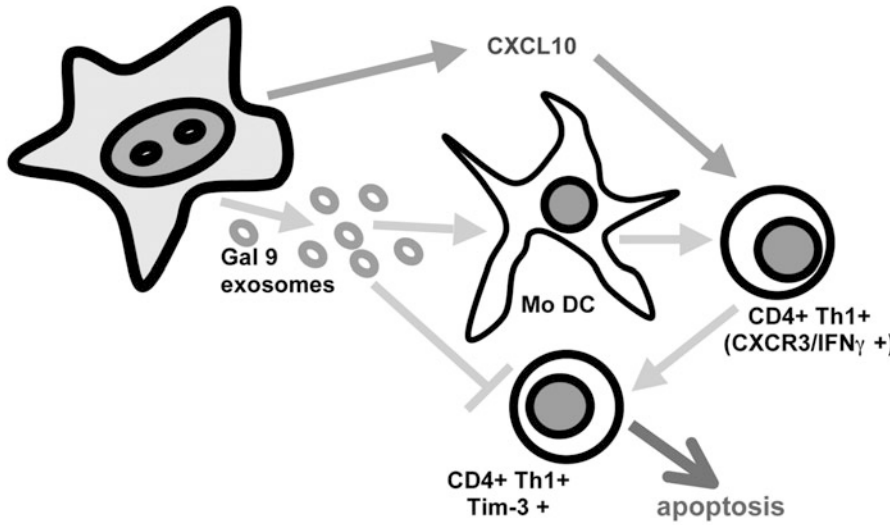

Figure 3. Hypothesis of an abortive maturation of CD4+ Th1+ lymphocytes in NPC tumor microenvironment. Tumor exosomes containing galectin 9 are expected to stimulate maturation of monocyte-derived dendritic cells (Mo DC) in a way that will favor differentiation and polarisation of Th1 lymphocytes.[90] CXCL10 secreted by tumor cells is expected to have a similar influence.[29] We hypothesize that additional maturation and polarisation of Th1 lymphocytes in situ results in Tim-3 expression . At this stage, Th1 lymphocytes will become vulnerable to the attack by galectin 9-positive exosomes and will be promptly eliminated.[89] If this scenario is true, Tim-3-positive CD4+ Th1 lymphocytes should not be detectable among NPC TILs; a point which remains to be investigated.

Evidence of Immuno-Editing

The concept of immuno-editing or immuno-selection in NPC tumors is supported by two series of observations. One series about EBV strain restriction in tumor tissue and the other about acquired resistance to apoptosis in malignant cells.

EBV strain diversity is consistently more restricted in tumor tissue than in saliva or circulating blood. In most cases, only one strain is present in the tumor. Remarkably, this unique tumor-associated strain often has genetic polymorphisms that selectively invalidate viral CTL-epitopes restricted in the HLA alleles of the patient.[91] At the single patient level, these data strongly suggest that genomes of certain viral isolates are selectively retained in the tumor cells for their ability to escape immune recognition. With regard to large groups of population, the same data suggest that combinations of HLA alleles and EBV-strain repertoires prevalent in certain geographic areas might favor a high incidence of NPCs.

Another form of immune-editing is suggested by a more aggressive phenotype of NPC tumors infiltrated by T-lymphocytes positive for granzyme B and perforin.[73] This observation suggests that intra-tumoral CTLs, although unable to eradicate all malignant cells, nevertheless select a subpopulation of tumor cells resistant to apoptosis. One possible consequence of these observations is that the immune response in NPC not only fails to block tumor growth but might result in an increase of NPC cell resistance to therapeutic agents.

CONCLUSION

In conclusion, it is interesting to address these two questions: what can we do to make further progress in this field? What will be the practical consequences of our knowledge of cellular interactions in NPC tumors?

Detection of cell population markers and cytokines on tumor tissue sections will have more and more importance in this field for the years to come. One important challenge is to achieve simultaneous detection of multiple targets on the same tissue section. One recent exciting trend in clinical investigations of NPC has been the discovery of a series of novel tumor markers in the peripheral blood of NPC patients.[53,92,93] Interestingly many of these markers are related to cellular interactions in NPC tumor microenvironment. [53,92,93] Their detection in the blood is related to their production in the tumor and provide indirect informations on cellular interactions. Several of these markers which have relevance to cellular interactions inside NPC tumors are listed in Table 2. In the coming years one may expect that some of them will contribute to assessment of intra-tumoral cellular interactions in combination with data obtained by immunohistology.

A major challenge is to connect observations made on clinical samples with in vitro experimental systems allowing assessment of functional roles of cytokines, exosomes and other cell communication agents. For this aim, novel methods allowing 3D in vitro cultures of cells derived from NPC biopsies need to be explored more intensively. In the past, some attempts to culture NPC cells in vitro under the form of spheroids have resulted in some partial success.[94,95]

In terms of therapeutic, there are several consequences of our knowledge of cellular interactions in NPC. Some therapeutic agents will be useful to block stimuli given by

infiltrating cells to malignant cells. For example inhibitors of the met-receptor which are currently in phase I or II trials might be useful to block tumor growth promoting effects by HGF released by stromal lymphocytes or fibroblasts.[60,61] Other therapeutic approaches will aim to antagonize the immunosuppressive effects of cytokines or exosomes released by malignant epithelial cells.[89] Finally based on our appreciation of cellular interactions in NPC tumor growth, it is useful to end with a word of caution about therapeutic strategies based on induction of the lytic cycle which might favour the release of a wide range of cytokines with potential enhancement of tumor growth and angiogenesis.

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CHAPTER 7

BIOLOGICAL TOOLS FOR NPC POPULATION SCREENING AND DISEASE MONITORING

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Abstract: Like for most human malignant diseases, it is essential to acquire biological tools for early diagnosis of small tumors, initial evaluation of tumor aggressiveness and rapid assessment of treatment efficacy. Because of the selective infection of the malignant NPC cells by the Epstein-Barr virus, most of the research on tumor biomarkers and other biological tools for NPC screening and monitoring has been oriented towards detection of antibodies against viral proteins or direct detection of viral products in the peripheral blood. NPC development is often accompanied by a rise in the titers of circulating anti-EBV IgG and de novo occurrence of anti-EBV IgA. Moreover these serological changes can occur prior to the onset of an invasive NPC. However the use of serum anti-EBV antibodies for NPC population screening has been hampered so far by their lack of specificity. Future progresses are expected to come from molecular analysis of the anti-EBV targets allowing selective detection of antibodies classes more closely related to NPC formation and development. Combination with other procedures like detection of viral RNAs in nasal swab might also be useful. Detection of plasma viral DNA by itself is not adequate for population screening because it is undetectable in a fraction of patients with small tumors. However, its initial pretreatment level has a pejorative prognostic value which is independent of the tumor extension. Moreover persistence of plasma EBV-DNA after treatment of the primary tumor is currently the strongest predictive factor of a pejorative evolution. Regarding long term follow-up, there are indications that periodic measurement of EBV-DNA loads might contribute to a more rational use of imaging techniques like PET-scan and therefore be cost-effective. Non-viral potential NPC biomarkers like circulating CCCL20, galectin-9 or cystatin-A also deserve further investigation.

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INTRODUCTION

The aim of this chapter is to summarize our knowledge on biological tools applicable to population screening, confirmation of diagnosis, prognostic evaluation, early assessment of treatment efficacy and posttherapeutic long-term surveillance. Most of these tools deal with a category of biomolecules which are usually called biomarkers. A biomarker is a biomolecule used as an indicator of a biological state. It is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. In oncology, based on the rational of high sensitivity and specificity rate, use of biomarkers is commonly admitted for some models (eg Alpha foeto protein for non seminomatous germ cell tumors), controversial in others (CA125 for ovarian cancer). The vast majority of other candidates are awaiting adequate prospective validation studies. While research on biomarkers is today extremely active, it is interesting to remember that for a long time specialists of NPC have been at the forefront of translational research on tumor biomarkers. At a time when our knowledge on molecular alterations of tumor cells was very limited, the presence of Epstein-Barr virus products in malignant cells was seen as a highly favourable feature for elaboration of biological methods for patient screening and monitoring. Moreover detection of EBV-related serological modifications in the plasma of NPC patients was the serendipitous observation which for the first time attracted attention to EBV involvement in this disease.[1,2] Soon after it was observed that modifications in EBV serology were often occurring prior to the onset of a clinically apparent tumor rising hope that EBV serology would allow rapid improvements in population screening and early diagnosis.[3] These promises have not been fulfilled mainly due to insufficient specificity of these serological alterations in endemic areas. Meanwhile, the range of potential biomarkers for NPC screening and monitoring has considerably broaden. In addition to detection of anti-EBV antibodies, this chapter will deal with detection of circulating viral products, especially EBV DNA and RNAs. We will provide some informations about nonviral circulating tumor products like cytokines and over-expressed proteins. We will briefly summarize the potential of direct biological exploration of the primary tumor and the surrounding nasopharyngeal cavity. Finally, the last paragraphs will attempt to summarize the most interesting applications of each biomarker and the potential of using them in combination.

CIRCULATING ANTIBODIES TO EBV-PROTEINS

In NPC patients, modifications of circulating antibodies to EBV-proteins are both quantitative and qualitative. They are not a direct reflection of tumor development but rather they reflect how the immune system reacts to viral products produced by tumor cells and maybe by other infected cells. By comparison with healthy EBV carriers, NPC patients consistently have higher titers of serum IgG against EBV proteins and de novo occurrence of anti-EBV serum IgA.[2,4-6] These antibodies are mainly directed against proteins of the lytic/productive cycle, for example, early antigens like Zebra/EB1 (BZLF1), the EBV-DNase (BGLF5), elements of the VCA complex like p18 (BRFR3), and gp350 (BGLF5).[7-10] This is probably a consequence of the disruption of latency occurring in a small fraction of malignant cells (see Chapter 4 by Gourzones et al). Remarkably, these serological alterations are often observed in advance to

tumor development, suggesting an increase of EBV replication in a premalignant context.[7,11,3,12-14] Antibody response against latent EBV proteins is mainly focused on EBNA1.[15] Serum antibodies against LMP1, LMP2, and BARF1 have been detected but they are inconsistent and with low concentrations.[16-19]

Several methods can be used to detect and quantify serum antibodies directed to EBV. The oldest methods are based on the application of increasing serum dilutions on fixed EBV-positive cells with a known pattern of EBV-antigens expression. Serum antibodies bound to target cells are revealed by fluorescent secondary antibodies (specific of either human IgG or IgA). Therefore, these assays are generally designated as immuno-fluorescent assays (IFA).[2,4] Antibody titers are calculated as the maximal serum dilution giving significant fluorescence under the UV microscope by comparison with a negative control. This method has been widely used for several decades but it has many drawbacks: it is labour intensive and requires skilled technical staff for making the slides (increasingly available form standardised commercial sources) and reading the results, which may be particularly difficult in low level responses such as IgA to VCA and EA. Importantly, IFA testing barely reveals the molecular diversity of antigen-recognition underlying antibody human polyclonal responses. This can be visualised by immunoblot analysis using well-defined extracts from EBV producer cell lines.[20,21] These studies have demonstrated that the molecular fine-specificity of anti-EBV IgA and IgG antibody responses in NPC patients is rather diverse between individuals as well as between IgG and IgA in the same individual and involves different EBV antigens and epitopes. This will have consequences for diagnostic test development. Multiple studies have been performed and are still in progress to design ELISA tests both reliable and affordable for NPC diagnosis and population screening. Commercial or hand-made ELISA tests based on native or recombinant proteins are still widely used.[22-24] However, there is a trend towards development of ELISA tests based on synthetic peptides which tend to be more specific and sensitive and easier to be manufactured on a large scale. For example, synthetic peptides carrying immunodominant epitopes present on the major antigenic EBNA1 and VCA/p18 proteins have been designed and validated in several studies (residues 382-410/413-452 and 119-148/153-176, respectively).[24-26] Fachiroh et al have used a test combining peptides derived from EBNA1 and VCA/p18 for detection of IgG and IgA in a large series of serum samples from healthy donors, nonNPC head and neck cancer patients and biopsy-proven NPC patients. The sensitivity and specificity for detection of combined anti-EBNA1/anti-VCA IgA in NPC patients was 95% and 90.6% respectively (defined by ROC analysis with positive and negative predictive values of 95.6% and 89.3% respectively).[25] The results were further improved by combination of the EBNA1/VCA ELISA with a second ELISA based on native EA protein.[26] In these studies like in studies made by other groups, the main concern remains to achieve an even greater specificity, because in various types of high risk populations, the number of individuals with EBV serological alterations is far greater than the number of subjects bearing detectable tumors.

DETECTION OF CIRCULATING VIRAL DNA

Detection of circulating extra-cellular EBV DNA was first reported in 1988 by Mutirangura et al using end-point PCR.[27] Very rapidly this new modality of biological exploration was considerably improved by application of real-time quantitative PCR using an internal fluorigenic probe.[28,29] In these initial studies, the levels of sensitivity and

specificity were remarkably good with EBV-DNA detected in the plasma of 96% NPC patients but only 7% of normal individuals. Furthermore, the EBV DNA concentrations were shown to be positively correlated with the clinical staging of NPC patients.

However subsequent investigations have given less clear cut results. Detection rates are variable in both NPC patients and healthy control subjects when looking at various reports on this subject.[30] These variations are related at least in part to patient selection and methodological differences. The detection rate is highly dependent on the tumor mass with a much higher detection rate for tumors with a large extension. This point is often overlooked. Nevertheless, it is well illustrated by comparison of the two following studies. Lin et al have reported detection of plasma EBV DNA in 94 of 99 patients with Stage III and IV but not in 40 healthy controls and 20 cured patients.[31] In contrast, Wei et al have reported detection of plasma EBV-DNA in only 61% of the patients affected by isolated recurrent primary tumors amenable to salvage nasopharyngectomy.[32]

Regarding methodological aspects, use of real-time PCR is crucial for sensitivity and specificity. Pre-analytical modalities of the preparation of plasma samples are very important.[30] Plasma seems to be a better starting material than serum.[33] High speed centrifugation is important to get rid of residual EBV-positive B-lymphocytes which are present in the blood of healthy EBV carriers and might occasion false-positive results. Quality control of DNA extracted from plasma samples is made by simultaneous amplification of a cellular target, for example the β-globin gene. Concentration of plasma EBV-DNA is generally expressed as the number of copies of the EBV genome per milliliter of plasma.[28,31] Calculation of the copy number is based on simultaneous amplification from an external standard made of plasmid DNA or DNA extracted from a cell line with a known number of EBV genome copies. In contrast to its major interest for prevention and management of posttransplant lymphomas, assessment of viral DNA from unfractionated whole blood has not proven to be very useful for NPC management.[34-36] First, there is detectable EBV DNA in whole blood of about 50% of healthy carriers.[35] Next, development of NPC tumors does not seem to be accompanied by an increase in the number of EBV-infected circulating B cells whereas circulating tumor cells do not seem to be very abundant in most NPC patients.[34,37] Overall detection of cell-free EBV-DNA in plasma is more specific and more sensitive for NPC patients.

At the analytical stage, the number of PCR cycles is a crucial factor. However, the increase in sensitivity resulting from increasing the number of cycles is accompanied by a certain decrease of specificity.[38] There is indirect evidence that plasma tumor DNA, including viral DNA, is released by apoptotic or necrotic tumor cells.[39] However, plasma EBV DNA does not seem to be carried by apoptotic bodies, but is apparently under the form of naked DNA fragments. Most of these fragments seem to be shorter than 181 bp.[40] Therefore the amplicon size resulting from primer design is another critical factor for sensitivity in detection of plasma EBV-DNA. In most publications, it is in the range of 60 to 120 nucleotides.[31,41] In theory, the PCR target sequence should be highly conserved without genetic variations across isolates. For example, one should be aware that the number of BamHI-W repeats varies in different EBV isolates. Practically this does not seem to be a major problem. Many authors use a target sequence from the Bam H1-W repeat which provides a somehow better sensitivity than unique EBV-genome sequences like the EBNA1 gene.[28,31,41,42] Recently, Lay et al have reported an interesting approach based on simultaneous amplification of 2 target EBV genes using a single plasmid containing both sequences as a quantification standard and the SYBR green dye instead of an internal fluorogenic probe.[43]

Overall, plasma EBV-DNA load appears as a remarkable biomarker for the management of NPC patients. There are still controversies about the extent of its possible applications.[34] Nevertheless, there is a growing consensus that assessment of circulating EBV-DNA is ill-suited for primary screening of NPC tumors. Its use for longitudinal posttherapeutic surveillance is a matter of debate (see section, *Patient Monitoring*, in this chapter). Conversely there is a growing consensus to think that circulating EBV-DNA is a very useful marker for early evaluation of treatment efficacy. In most cases, radiotherapy or concurrent chemoradiotherapy induces a dramatic decrease in plasma viral DNA load. In a majority of patients, the EBV DNA will decrease to zero copy in days following the completion of the treatment.[31,44] An even more rapid decrease is observed after salvage surgery for recurrent or persistent tumors in the nasopharynx or in lymph nodes.[32,45] The median half-life of plasma EBV DNA after surgical resection of NPC is less than 3 hours.[45] Persistence of circulating EBV-DNA is often associated with positive surgical margins indicating incomplete resection.[32] However, salvage surgery for NPC is a relatively rare procedure. Going back to radiotherapy or concurrent radio-chemotherapy, it is remarkable that persistence of detectable plasma EBV-DNA in the days following the completion of radiotherapy is highly pejorative in term of disease-free and overall survival, regardless of a low or high initial level of viral DNA.[31,44,46,47]

Several groups have investigated the kinetics of early changes in plasma EBV DNA under treatment by radiotherapy and/or chemotherapy. In a series of patients with metastatic or recurrent NPCs, Wang et al have found that the T1/2 of plasma EBV DNA clearance ranged from 1.85 to 28.29 days (median 3.99) (T1/2, time required for observation of a 50% reduction of EBV DNA load). Patients with a short T1/2 had significantly higher complete response rate and overall survival than those with long T1/2. One of their conclusions was that changes of chemotherapy regimens might be considered for patients with slow plasma EBV DNA clearance rate.[48] Similar results were reported by Hsu et al.[49] In the same vein, it is noteworthy that there is a consistent, transient rise of plasma EBV DNA in the days following the onset of radiotherapy with a peak occurring at day 3 after the initiation of treatment.[50] It would be interesting to know whether the amplitude of this transient early rise of plasma EBV DNA could be predictive of the long term tumor response to a given therapeutic combination, with the potential of being used as a pharmacodynamic marker.

DETECTION OF CIRCULATING VIRAL RNAs AND PROTEINS

The EBV genes which are most actively transcribed in NPC encode two types of small untranslated RNAs: (1) the EBERs and (2) the BART microRNAs (see Chapter 4 by Gourzones et al). EBER1 and 2 are made of about 17O nucleotides forming a single strand which is folded in a complex 3D structure containing loops connected to a central stem. The EBERs are strongly bound to ribonucleoprotein particles which make them relatively resistant to the action of various RNAses. In contrast the BART microRNAs are regular microRNAs encoded by the viral genome. Interestingly both the EBERs and the miR BART are consistently detected in plasma or serum samples from NPC patients.[27,31,51-53] Experiments performed in nude mice xenografted with NPC tumor lines strongly suggest that at least a substantial fraction of these circulating BART microRNAs are produced by tumor cells (see Fig. 1).[51] There are a number of questions which have not been yet answered about circulating viral microRNAs in NPC

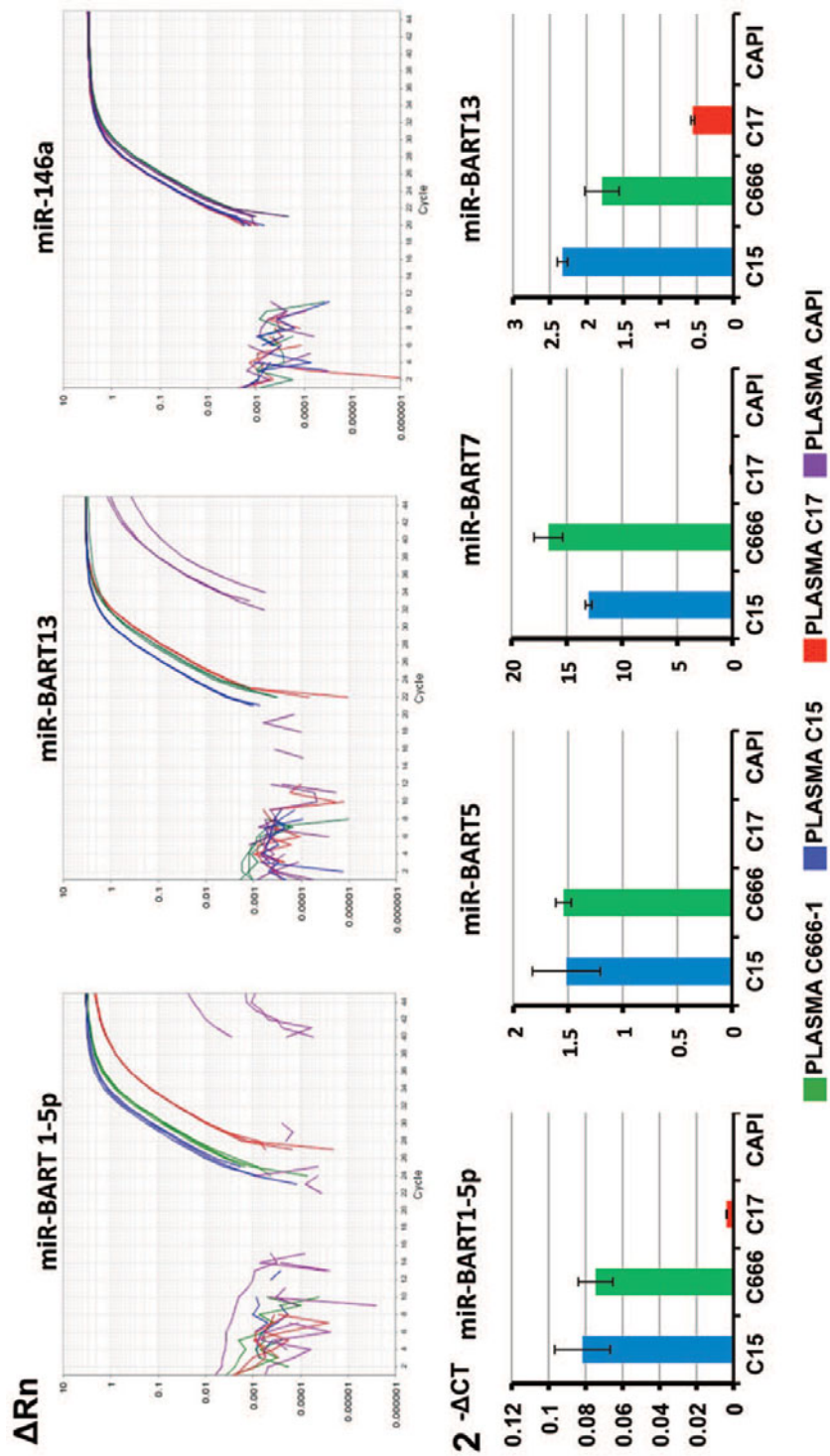

Figure 1. Please see the figure legend on the following page.

Figure 1, viewed on previous page. Examples of detection of EBV BART microRNAs in plasma samples from mice carrying xenografted NPC tumors (C15, C17, C666-1). These experiments have provided the proof of concept for investigation of EBV BART microRNAs in plasma samples from NPC patients.[51] Plasma samples were collected from mice xenografted with 3 EBV-positive nasopharyngeal carcinoma tumor lines (C15, C17 and C666-1) and from control mice xenografted with an EBV-negative human epithelial tumor (CAPI). Four EBV BART microRNAs—miR-BART1-5p, 5, 7-3p and 13—were detected by real time PCR following RNA extraction and reverse transcription. For each type of xenografted tumor, PCR analysis was performed on pools of plasma samples collected from 3 or 4 mice. The cellular miR-146a which is known to be detectable in blood plasma was used as an endogenous reference.[100] Upper panel) amplification plots obtained for miR-BART1-5p and 13 and for miR-146a. ΔRn stands for the magnitude of the fluorescence signal generated during the PCR at each time point (with background correction). Lower panel) histograms presenting the $2^{-\Delta CT}$ values for miR-BART 1-5p, 5, 7-3p and 13 (miR-146a being the endogenous reference). All 4 BART microRNAs are relatively abundant in plasma samples from mice xenografted with C15 and C666-1 whereas they are at a low level in samples from C17 mice. This reflects the lower abundance of BART microRNAs in the corresponding C17 tumor cells. Like for tumor RNAs directly extracted from the xenografted tumor, the $2^{-\Delta CT}$ index is several times higher for miR-BART 7-3p than for other BART microRNAs. Following these experiments on xenografted tumors, we were able to detect BART microRNAs, especially miR BART 7-3p, in plasma samples from NPC patients. On average, higher concentrations were observed for NPC patients than for healthy EBV carriers.[51] Figure reproduced from Gourzones C et al. Virol J 2010; 7:271;[51] with permission under the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0).

patients. Is their concentration proportional to the tumor mass? Are they detectable in the plasma of patients with small tumors? By which specific carriers are they protected from the action of plasma RNAses? This last question has been addressed by several investigators interested in the biology of circulating microRNAs in healthy subjects or in various pathological conditions. So far, at least four types of microRNA carriers have been identified in human plasma: microvesicles, exosomes, High Density Lipoproteins (HDL) and ribonucleo-protein particules containing the argonaute 2 protein.[54-57] Exosomes are bi-lamellar nanovesicles of 30 to 100 nm in diameter which are secreted by many cell types and derive from structures of the endosomal pathway called multivesicular bodies. Exosomes contain various types of cellular proteins which are either luminal or membrane-inserted.[58] In contrast, microvesicles which have a diameter of 100 nm to 1μm arise by burgeoning of the plasma membrane.[59] Both exosomes and microvesicles carry nucleic acids including messenger RNAs and microRNAs.[60,61] Preliminary data obtained by our group suggest that EBV microRNAs detected in the plasma of NPC patients are not predominantly associated with circulating exosomes, microvesicles or high density lipoproteins (C. Gourzones and P. Busson, unpublished data). The possibility of their association with ribo-nucleo-protein complexes will deserve further investigations. One group has reported the detection of the BARF1 and LMP1 EBV-proteins in plasma samples from NPC patients but not from healthy controls. BARF1 was in a soluble form whereas LMP1 was apparently contained in circulating exosomes.[62] Circulating exosomes containing LMP1 have also been detected in mice xenografted with NPC tumor lines.[63]

DETECTION OF CIRCULATING NONVIRAL TUMOR PRODUCTS

Although it is much easier to assert the tumor origin of viral nucleic acids in biological fluids, release and blood diffusion of tumor DNA and RNA is not restricted to those of viral origin. For example, cellular DNA fragments containing hypermethylated promoters of cellular genes like CDH1, DAPK, and CDKN2A are detected in plasma samples from a majority of NPC patients.[64]

Regarding serum or plasma cytokines, consistent alterations are an increase in the concentration of soluble CD23 (sCD23), TGF-β, interferon-γ, soluble CD40-ligand (sCD40L) and CCL20 (also called MIP3-α).[65-70] There is strong evidence that a large fraction of sCD23 and CCL20 detected in plasma samples are derived from the malignant cells. A high concentration of plasma sCD23 has a pejorative prognostic value for initially nonmetastatic, locally advanced NPC patients, resulting in an increased risk of local relapse.[66] According to Chang et al, the serum concentration of CCL20 is correlated to the tumor mass and is predictive of a higher risk of metastatic recurrence, independently of the clinical parameters.[67,71] Simultaneous assessment of multiple cytokines detected in the peripheral blood of NPC patient will probably improve the power and accuracy of patient prognostic classification.[71] Non-cytokine proteins derived from malignant cells are also consistently detected in the serum of NPC patients, for example the cellular protein cystatin A which is associated with a higher nodal stage in NPC patients.[72]

Our group has shown that tumor exosomes are consistently detected in the plasma of NPC patients and represent a potential source of protein-based biomarkers (in addition to nucleic acids). Remarkably, NPC exosomes have a high content of HLA class II molecules.[73] Magnetic capture using beads coated with anti-HLA class II is a powerful tool to capture tumor exosomes from the plasma of NPC patients. This capture method has a good specificity for NPC tumor exosomes, since no exosomes are captured from control plasma samples in the same experimental conditions.[74] In addition to HLA class II proteins, NPC exosomes have a high content of galectin-9. Galectin-9 is a β-galactosyl binding lectin, which is very abundant in NPC cells and known to have immunosuppressive properties.[75,76] Even based on immunomagnetic capture, isolation of plasma tumor exosomes remains labor-intensive and time-consuming. However, in the future, one might consider direct detection of galectin-9 in whole plasma samples using a highly sensitive ELISA test.

BIOLOGICAL EXPLORATION OF THE PRIMARY TUMOR AND SURROUNDING NASOPHARYNGEAL CAVITY

Currently, nasopharyngeal (NP) biopsy remains the gold standard procedure for diagnosis of NPC. Fine needle aspiration combined to detection of EBV DNA is useful when dealing with a metastatic lymph node of an unknown primary but not for the diagnosis of a primary nasopharyngeal tumor.[77] Following tumor biopsy, pathological identification of carcinoma-type proliferation is usually confirmed by EBER RNA in situ hybridisation (RISH). In addition immunohistochemistry on tissue sections of the primary tumor allows detection of multiple viral and cellular proteins. Non-viral proteins of interest, can be observed not only in malignant cells but also in stromal cells or infiltrating lymphocytes.[70,78,79] Due to the high sensitivity and specificity of EBER detection (RISH), detection of EBV-proteins like EBNA1 (Epstein-Barr nuclear antigen 1) or LMP1 (latent membrane protein 1) has no significant impact in terms of diagnosis. So far, despite numerous promising initial reports, no single cellular protein detectable by immunohistochemistry has gained wide acceptance as an independent prognostic marker. For example, the prognostic value of LMP1 detection by itself is a matter of controversy.[80-83] However its detection might find novel justifications with the advent of immunotherapy protocols targeting LMP1.[84] Loss of pro-caspase 3 expression, abundance of nuclear survivin or high expression levels of the c-met receptor in the malignant NPC

cells are other examples of primary tumor characteristics which have been reported as predictive of pejorative outcome but—to our knowledge—are not currently used for patient management.[79,85,86] Progress is likely to result from simultaneous assessment of the expression levels of several proteins detected in tissue sections from the same tumor. A recent report by Wang et al (2011) has exemplified this type of approach. Using tissue microarrays (TMA) and a semi-quantitative staining score, they have identified a prognostic classifier based on eight viral and cellular proteins including LMP1, CD147, caveolin-1, matrix metalloproteinase 1, survivin and SPARC (secreted protein acidic and rich in cystein). In multivariate analysis adjusted for age, TNM stage and histological subtype, this classifier is an independent predictor of survival which allows classification of patients either in a low or in a high risk group with a significant difference in 5-year survival.[87]

Although it is a key, indispensable step for management of NPC patients, nasopharyngeal biopsy remains an invasive procedure which requires an operator competent in retro-nasal endoscopy, generally an ENT surgeon. Therefore there is a need for additional procedures—based on novel imaging techniques or/and novel types of biological explorations—in order to improve exploration of the primary tumor and surrounding space in the nasopharyngeal cavity. One long term aim is to reduce the number of NP biopsies especially in the context of population screening. Interesting examples of these novel approaches are procedures based on nasopharyngeal swap (also called nasopharyngeal brushing). A sterile swab or brush is passed gently through the nostril and into the nasopharynx with the patient's head slightly tilted back to straighten the passage from the front of the nose to the nasopharynx, generally under local spray anesthesia. Then the swab is gently rotated against the mucosa. This procedure is often used for bacterial or viral diagnosis of infectious diseases (diagnosis of B. Pertussis, influenza, etc…). In the case of NPC patients, it allows collection of material released by the NPC primary tumor and surrounding mucosa or—in the setting of population screening—by the premalignant mucosa. Initially, end-point PCR was used in several studies combining nasopharyngeal brushing and detection of EBV DNA in collected samples.[88-90] All these studies demonstrated that EBV-DNA was readily detected in samples from the vast majority of patients with biopsy-proven NPCs (often in about 90% of these patients). However EBV-DNA was also detected in NP swabs from a fraction of nonNPC individuals varying from 20 to 50%. More recently, using real-time PCR, Stevens et al have confirmed that EBV DNA is detectable in NP brushings from 100% NPC patients (most of them with large tumors) and about 80% of nonNPC cases but with striking differences in the copy numbers.[91] The very high copy numbers obtained in samples collected from NPC patients by comparison with controls has allowed establishment of cut-off values which result in very good values for sensitivity, specificity, positive and negative predictive values.[92] In addition, using semi-quantitative techniques, the same group was able to detect EBV messenger RNAs by RT-PCR in samples from about 80% of NPC patients but not in any sample from control individuals.[91] The fact that abundant transcripts of lytic genes are detected in NP brushings from NPC patients suggest that viral replication and virion release is taking place at the surface of the tumor (Greijer et al, poster presentation PP4, 5th International Symposium on Nasopharyngeal Carcinoma, Penang, Malaysia, June 22nd-24th, 2011). This group has also used DNA from NP brushings for detection of methylation on promoters of tumor suppressor genes. They have reported a combination of five methylation markers (RASSF1, p16, WIF1, CHFR and RIZ1) which gives good discrimination between NPC and nonNPC samples.[93] In summary, NP swab or brushing appears as a promising approach for future facilitation of NPC diagnosis. It

offers the possibility to combine a non-invasive clinical procedure with various types of molecular biology investigations. In the most recent studies, the brushing was guided by naso-endoscopy.[91] For future applications of this procedure in population screening, it will be interesting to know whether similar results are obtained without visual control especially when dealing with small tumors. Another important question will be to know whether nasal swabs may contribute to detection of genetic or epigenetic changes in premalignant mucosa not yet infected by EBV[94] (see Chapter 5 by Lo et al).

CURRENT APPLICATIONS AND PERSPECTIVES

Population Screening

For NPCs as for many other cancers, presence of a large tumor mass is a major pejorative prognostic factor. Improving rates of early detection, early diagnosis and early treatment is expected to reduce the burden of this disease in terms of mortality, morbidity and long term sequels. This is a major challenge because even in regions with elevated rates of NPC incidence, this disease remains relatively rare and because no current biomarker is fully satisfactory for this task. As previously mentioned, detection of circulating EBV DNA is not adequate for population screening at least by itself.[42,95] Almost all investigations in this field are based on EBV serology (see Table 1). Two types of approaches have been undertaken to assess the feasibility and the potential of population screening.

One approach deals with the general population in high incidence areas. Such very large prospective screening studies have been performed in southern China and in Taiwan often with population numbers in the range of 10,000 to 20,000.[7,12-14] They have confirmed that alterations in the profile of circulating EBV-antibodies—especially detection of IgA against VCA—are either concomitant of the development of small size NPC tumors or predictive of a higher risk of tumor development in the subsequent months or years. Among subjects with circulating anti-VCA IgA, the first year detection rate of NPC is about 31 times greater than the incidence of NPC in the general population for the same age group. In the subsequent 4 years, it remains 7.5 times higher than in the general population for the same age group.[12] However, serological screening is hampered by a major lack of specificity. Indeed in endemic areas, a relatively large fraction of the population—varying from 1% to 10%—has alterations of the serum anti-EBV profile. Among them, only a very small minority will develop a tumor, 4% at a maximum.[7,14] Most individuals with elevated anti-EBV IgA will undergo normalisation of their serologic profile in a few months or years without any pathological manifestations. Rise of anti-EBV antibody titers at two distant blood collections is associated with an increased risk of tumor development whereas a serological normalization or descending titers are associated with a reduced risk.[14,13] However, on average, all subjects who have presented elevated anti-EBV IgA at once remain at a higher risk than the general population.[7,14] Current studies intend to strengthen these results by comparison with better control groups. For example, they randomize individuals in two series: (1) one with periodic blood collections, serological profiling and nasopharyngeal explorations requested on the basis of serological abnormalities and (2) a control series whose people only undergo periodic interviews about possible symptoms of NPC and nasopharyngeal explorations motivated by suspicious symptoms.

Table 1. Main NPC diagnosis and predictive biological markers classified according to their interest through the various stages of patient care

Stages of Patient Care	Preventive Population Screening	Initial Diagnosis	Pronostic Evaluation (Risk Stratification)	Early Assessment of Treatment Efficacy	Long-Term Posttherapeutic Surveillance
Main pathological events or conditions for which diagnosis or prediction are desired.	- Abnormal EBV replication in the nasopharyngeal cavity (or another anatomic site?) - Premalignant alterations of the nasopharyngeal mucosa - Occult primary tumor	Clinically visible primary tumor	Occult visceral metastases	All malignant lesions	Metastatic recurrence
Biological tools and markers which are currently available.	Detection of anti-EBV IgA, for example anti-EBNA1 and anti-VCA-p18[11,25]	- Histological. diagnosis - EBER in situ hybridization	Re-treatment level of plasma EBV-DNA load[96]	- Presence or absence of circulating EBV-DNA at the completion of the radiotherapy or radio-chemotehrapy (EBV-DNA persistence is highly predictive of an early metastatic relapse).[31,44,46,47] - Clearance rate of circulating EBV-DNA through the duration of the initial treatment (~ first 12 to 24 weeks)[48-50]	Resurgence or de novo detection of circulating EBV DNA[99]
Potential alternative or complementary investigation tools (including imaging modalities)	- Nasal swap or brushing combined to detection of viral DNA or RNA or methylated cellular DNA[91,93] - Detection of novel types of anti-EBV antibodies against defined viral protein epitopes[25,26] - Detection of circulating viral microRNAs or EBERs[51]		- Immunophenotype of malignant and stromal cells on tissue sections of the primary tumor[87] - Profile of circulating cytokines and other proteins released by malignant or stromal cells[71,72]	Transient rise in the concentration of circulating EBV DNA in the days following the onset of the treatment[50]	PET scan[99]

Another approach of NPC population screening deals with selected groups of individuals within populations of high incidence areas, for example families with multiple cases of NPC. The seroprevalence of anti-EBV VCA and EBNA1 IgA is much greater among healthy individuals from high-risk NPC multiplex families than among healthy individuals from the general population in endemic areas.[23] A study by Yu et al has shown that the risk of NPC is increased almost 7 fold for individuals with high levels of anti-EBNA1 IgA using a cut-off point optimised by ROC analysis.[11] In contrast to previous studies on sporadic NPCs, the anti-EBNA1 IgA level—assessed by ELISA on the recombinant protein—was a better marker than the anti-VCA IgA level. In conclusion of their study the authors pointed out a lack of specificity which is summarized by the following observation. Above the threshold of anti-EBNA1 titers required to pick up 90% individuals who developed NPC, 50% individuals who did not develop NPC also tested positive. This remains a general problem in the use of serologic markers for early detection of NPCs.

Another type of selected groups can be defined on the basis of clinical criteria. They gather individuals with symptoms and signs suggestive of NPC. Recruitment of such patients can be stimulated by prevention campaigns in high incidence areas to make people better informed of the risk of NPC and revealing symptoms (nasal blockage, nose bleeding, blood stained saliva, hearing loss…). These preselected patients are then subjected to a series of diagnostic procedures which are graded according to their costs and their more or less invasive nature. Decision algorithms combining EBV serology, measurement of EBV DNA load, CT scan and nasal brushing prior to nasopharyngeal endoscopy and biopsy are currently tested in Indonesia (J.M. Middeldorp, oral communication, 5th International Symposium on Nasopharyngeal Carcinoma, Penang, Malaysia, June 22nd- 24th, 2011).

In conclusion, application of NPC biomarkers to early tumor detection is likely to progress through the convergence of the following innovative approaches: (1) molecular analysis of anti-EBV response and refinement of immunological detection tools, for example ELISA on novel synthetic peptides; (2) combination of anti-EBV antibody detection with other biomarkers, for example detection of circulating microRNAs or EBV-RNAs or methylated DNA in nasal brushings; (3) combination of biomarker investigations with imaging techniques.[8,25,42,95]

Patient Monitoring

NPC diagnosis relies almost entirely on tumor biopsy combined to EBER detection as explained in paragraph F. In the near future, initial determination of the prognosis or risk stratification will probably mainly benefit from two types of biological investigations: immunophenotyping of the primary tumor and measurement of the plasmatic EBV DNA load[87,96] (see Table 1). The pretherapeutic level of plasma EBV DNA is a prognostic for overall survival which is independent of the tumor extension. Leung et al have defined a cut-off value of viral DNA load which allows attribution of a low or high risk to patients with Stages I and II as well as patients with Stages III and IV.[96] In contrast, serum levels of anti-EBV antibodies are of poor predictive value.[97,98] As mentioned in a previous paragraph, assessment of pretherapeutic levels of selected groups of cytokines is also a promising approach which will probably benefit from additional validation studies.[71]

Regarding assessment of treatment efficacy, as already mentioned, the persistence of plasma EBV DNA after initial treatment (radiotherapy or concomitant chemoradiotherapy) is a very strong predictor of worse outcome and of rapid distant metastatic relapse.[31,44,46,47] According to a growing number of investigators, persistence of plasma EBV-DNA after the initial treatment seems sufficient to invite additional therapeutic action (see Chapter 10 by Hui and Chan). Moreover, a low clearance rate of EBV DNA evaluated by repeated assays during the first weeks of treatment is also of pejorative value.[48-50] In the future, it might advocate therapeutic adjustments. Again use of anti-EBV serum antibodies for treatment monitoring seems less promising; their clearance rate being usually much lower.[98]

Finally regarding long term posttherapeutic surveillance, it is not yet clear whether any biological tool can contribute to early detection of recurrence in addition to clinical and imaging monitoring. However a recent study shows that a longitudinal follow-up based on periodic viral DNA testing (every 3 to 6 months) and FDG-PET (Fluoro-deoxy glucose—Positron Emission Tomography) in case of positive conversion is efficient and cost-effective by comparison with a follow-up based on clinical surveillance and systematic annual FDG-PET exploration.[99]

CONCLUSION

Molecularly defined serology and detection of viral RNAs in nasal swap or brushing are currently the most promising tools for NPC population screening. On the other hand, detection of plasma EBV-DNA is gaining recognition as an interesting tool for improving patient monitoring.

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CHAPTER 8

MEDICAL IMAGING OF NASOPHARYNGEAL CARCINOMAS: Current Tools and Applications

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Abstract: Imaging of nasopharyngeal tumors is crucial for staging, preparing and evaluating treatment and for ensuring follow-up. Computed tomography, magnetic resonance imaging and fluorodesoxyglucose positron emission tomography are the main imaging tools. Each device is endowed with its own advantages for local, regional or distant staging and during medical care.

INTRODUCTION

Imaging of nasopharyngeal tumors is crucial in daily practice. The physician has to attain four objectives: stage the tumour and correctly describe its extent, evaluate treatment and be aware of the usual posttherapeutic appearance, recognize a recurrence. This chapter reviews the imaging devices usually used which are computed tomography (CT), magnetic resonance imaging (MRI) and fluorodesoxyglucose positron emission tomography (FDG-PET), and their use during medical care.

Nasopharyngeal Carcinoma: Keys for Translational Medicine and Biology,
edited by Pierre Busson.

IMAGING DEVICES

Nowadays, plain films of the head and neck are no longer used for nasopharyngeal cancer management. Plain films of the chest, liver ultrasound and bone scintigraphy tend to be replaced. CT, MRI, and 18F FDG-PET are currently the main and adjunct tools used to explore nasopharyngeal cancer.

CT is routinely performed and is often the baseline imaging examination worldwide. This technique generates a three-dimensional image of X-ray attenuation coefficients in the human body. CT is a great tool for cortical bone analysis and is also quite good for soft tissue analysis (ranking second to MRI). Its advantages are a short acquisition time (shorter than MRI) and the avoidance of motion artefacts. When a nasopharyngeal tumor is explored for the first time, a chest CT is often also performed to search for metastases. Even if CT is the baseline imaging examination, it has been challenged for years by MRI and FDG-TEP in most of the publications and in daily clinical practice.

MRI is also routinely performed. This technique generates different sets of images of the anatomy thanks to the magnetic properties of hydrogen in different tissues. The sets of images acquired are named T1-weighted, T2-weighted for instance, which refer to relaxivity properties (Fig. 1). The main advantages of MRI are: its great ability to depict tumor in soft tissues, bone marrow, along nerve paths and in the cerebral cavity.

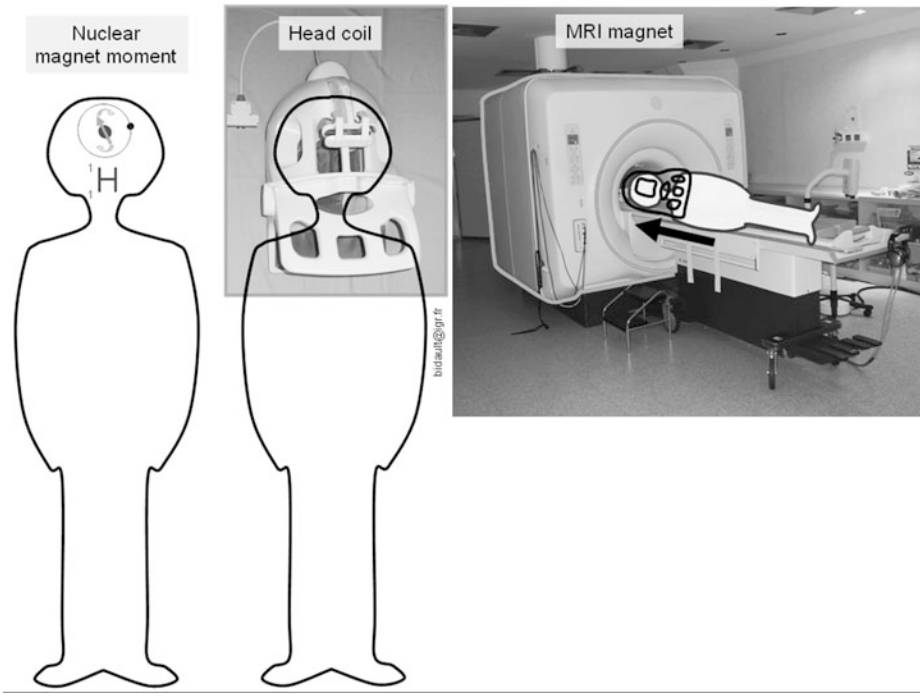

Figure 1. Magnetic properties of hydrogen are explored thanks to dedicated coils and radio frequency waves. To do so patient and coils have to be placed in the high magnetic field generated by the machine (routinely 1.5 to 3 Tesla).

The main drawbacks of MRI are: its sensitivity to patient motion and metallic artefacts. The main contraindications for MRI are due to the high magnetic field generated by the machine: pacemakers, other implanted electronic devices, metallic orbital foreign bodies, some vascular clips and cardiac valves for instance. According to AD King et al, MRI demonstrates great sensitivity (100%) in depicting nasopharyngeal cancer (it is superior to endoscopy without a biopsy) and lower specificity (93%) than an endoscopic biopsy.[1] Some improvements of MRI such as diffusion-weighted imaging (DWI) are also described in the literature. It is able to depict the movement of water in the extracellular compartment. This mobility is usually decreased in tumour tissues. Like other imaging techniques, DWI has not demonstrated its ability to replace the pathological analysis.[2]

For both MRI and CT, contrast-enhanced acquisitions are routinely performed and recommended, with intravenous injection of contrast media: iodinated and Gadolium-based contrast agents for CT and MRI respectively.

FDG-PET is a nuclear medicine imaging technique that produces a three-dimensional image of FDG tracer uptake. Images provide a glimpse of tissue metabolic activity in terms of regional glucose uptake, quantified with a standard uptake value (SUV). The FDG tracer is injected intravenously. As head and neck undifferentiated and squamous cell carcinomas are avid for glucose, FDG-PET is a good tool for (1) assessing nodal involvement and distant metastasis, (2) searching for the primary tumor when the physical examination, CT and MRI only depict pathological nodes, and (3) ensuring patient follow-up.[3] Improvements of PET concern devices and also vectors such as 11C-Choline to enhance T delineation which is usually limited with an FDG examination due to physiologic uptake in the brain.[4]

All these devices may face some pitfalls that result in a small rate of false positive and false negative results for the tumour diagnosis.

IMAGING DURING MEDICAL CARE

The role imaging plays during staging and its impact on treatment is well established in daily practice and described in the literature.[5,6]

Nasopharyngeal tumour staging is according to the UICC classification (currently the 7th version). Imaging cannot replace the pathological analysis for the diagnosis of cancer. Moreover, a T1 tumor and normal nasopharyngeal mucosa can look quite similar on imaging. The main benefits of medical imaging are: to depict T2, T3 and T4 stages, to describe the extent of nodal involvement (especially retropharyngeal nodes), and the metastatic status. Thanks to its good soft tissue contrast, MRI demonstrated early primary tumor involvement more precisely than CT.[7] Furthermore, MRI and CT are the best tools to describe the T stage, whereas the use of FDG-TEP is nowadays well established for N and M staging[8] (Fig. 2). Both MRI and CT depict invasion of the parapharyngeal space leading to Stage T2 (Fig. 3). For Stage T3, CT displays cortical bone involvement, whereas MRI is superior in showing medullary bone invasion. MRI is better than CT for depicting intracranial perineural spread (along the V3 cranial nerve for instance) leading to the T4 stage (Fig. 4).

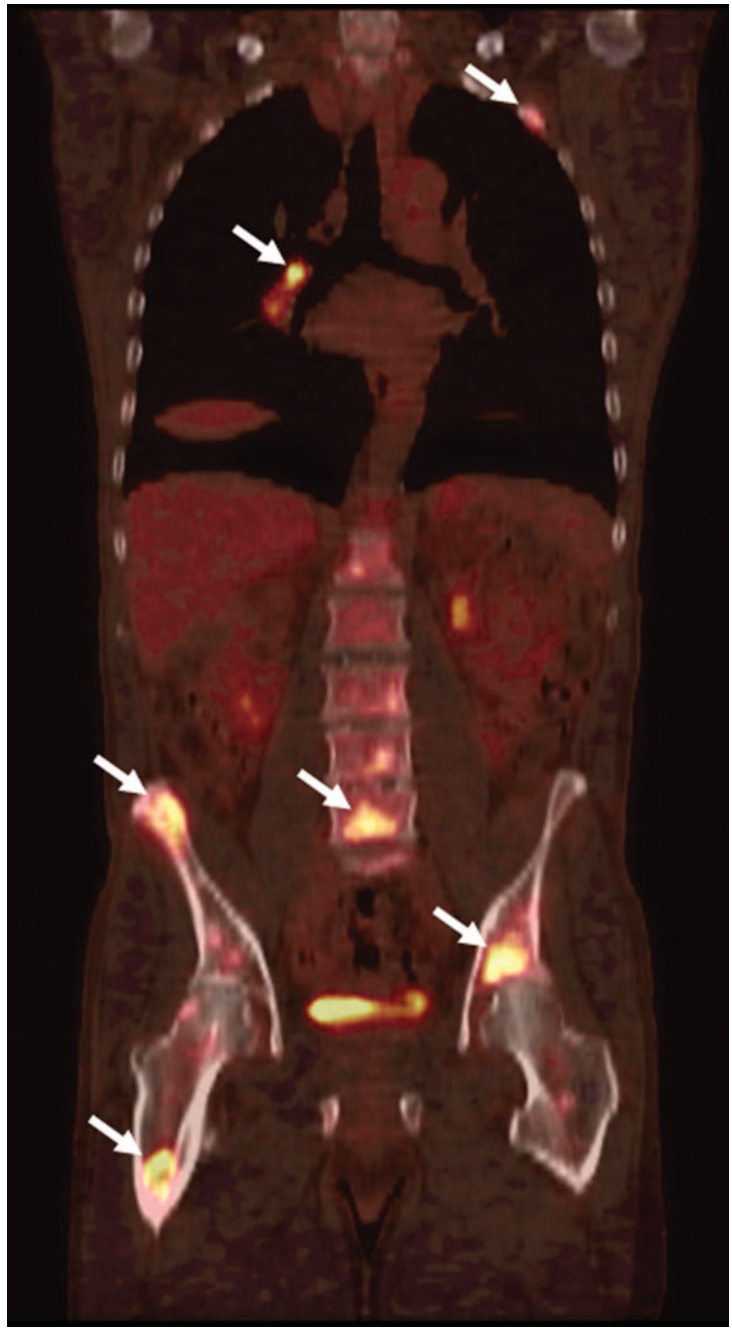

Figure 2. FDG-PET depicts distant metastasis (arrows).

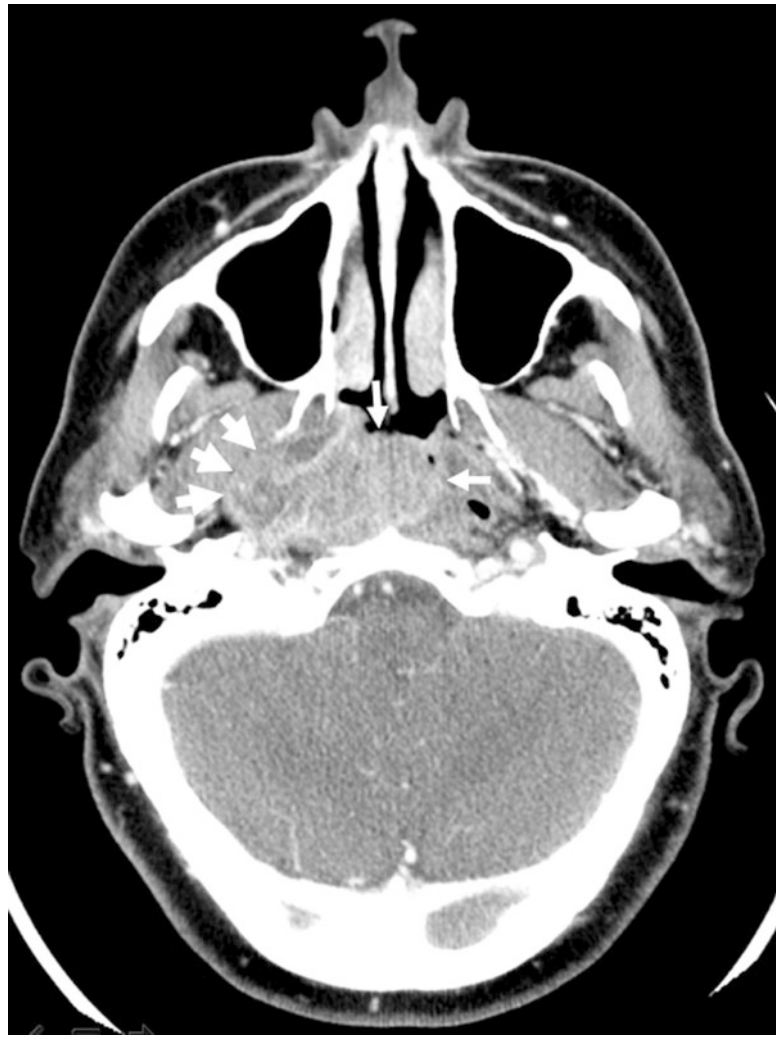

Figure 3. Contrast-enhanced CT shows parapharyngeal extension (large arrows), T2 according to the UICC classification (currently the 7th version).

IMAGING AND TREATMENT

Imaging is an important tool for preparing treatment and assessing treatment efficacy. Imaging completes the physical examination aimed at assessing tumor response to first-line chemotherapy. A decrease in tumor size (diameter or volume) after first-line chemotherapy can be visualized on CT or MRI and a decrease in tumour metabolic activity can be shown with FDG-PET. A dedicated CT scan (performed in the treatment position) is also recommended and performed in daily practice to plan external beam radiotherapy. In addition, teams are testing the impact of other imaging devices (FDG-PET and MRI) when planning radiotherapy.[9,10]

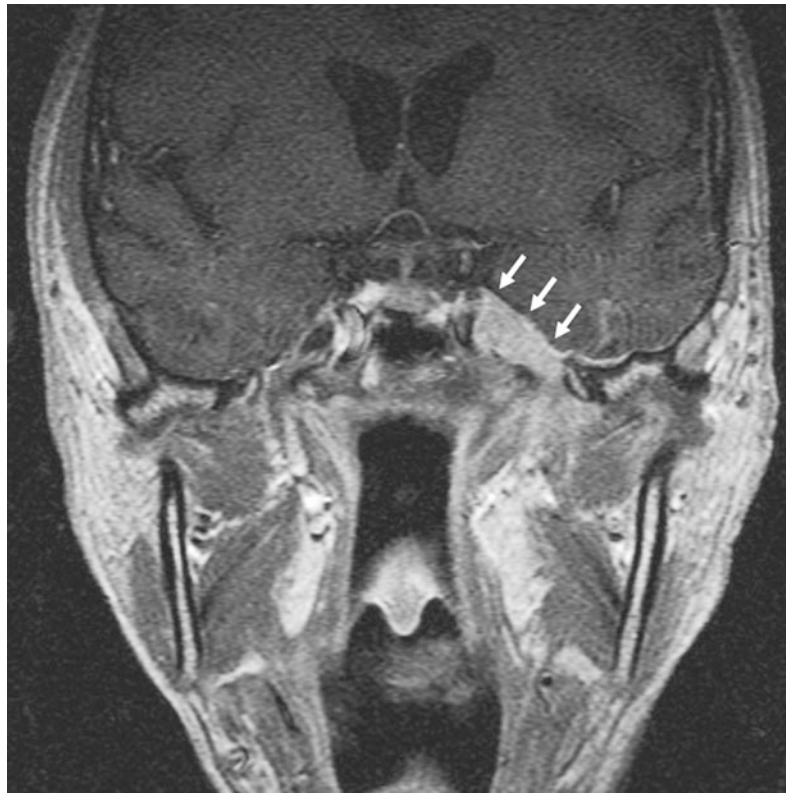

Figure 4. Contrast-enhanced T1-weighted MR Imaging depicts intracranial perineural spread along the V3 cranial nerve (arrows).

IMAGING AND FOLLOW-UP

There is a paucity of guidelines about imaging during follow-up. Some recommendations and study results can however be presented. CT, MRI and FDG-PET can be performed during follow-up. For local follow-up, MRI tends to yield greater accuracy than FDG-PET.[11] On the other hand FDG-PET, recommended for its negative predictive value after treatment, has been reviewed as the best follow-up imaging device compared to CT and MRI.[12,13] Some improvements in MRI have enabled it to compete with FDG-PET for treatment follow-up with similar sensitivity and specificity.[14]

In daily practice MRI and/or CT can be recommended as the baseline follow-up examination at 3 months after treatment completion. The aim of this examination is to identify anatomy alterations due to the tumor and treatment. These alterations can lead to interpretation difficulties when a recurrence is suspected.[15] Comparing the baseline follow-up examination with subsequent studies can help differentiate simple posttherapeutic alterations from recurrence.

One posttherapeutic complication, namely cerebral necrosis, must be identified because it can be confused with a brain metastasis. This alteration occurs exclusively in irradiated areas which are often the temporal lobes in nasopharyngeal cancer. This alteration grows and

then decreases over many months and may lead to an erroneous diagnosis of a recurrence during the increasing phase. The patient's history, imaging features and some advanced MRI sequences (permeability/perfusion, proton spectroscopy) may lead to the correct diagnosis of radionecrosis. A residual neck mass after treatment can also be troublesome: is it recurrent tumor or simply a posttherapeutic alteration? In this case, CT and fine needle aspiration (FNA) have a poor negative predictive value for recurrent nodal disease in NPC.[16] FDG-PET is actually a good tool for its solid negative predictive value in this case.

When a recurrence is highly suspected, the pathologist will be needed most of the time to decide whether further treatment is required. Rarely will a core biopsy not be technically feasible due to the deep location of the suspected recurrence (for example, skull base lesions).

CONCLUSION

Imaging is an adjunct to physical examination to stage nasopharyngeal cancer, assess tumour response to treatment and ensure follow-up. CT, MRI and FDG-PET are the main tools. All of them have their own advantages and drawbacks and are still in progress.

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CHAPTER 9

RADIOTHERAPY OF NPC: Current Strategies and Perspectives

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Abstract: Radiation therapy (RT) remains the mainstay of treatment for NPC patients without evidence of metastases. The goal of radiation therapy is to cure patients while preserving normal tissue function. Results from randomized clinical trials support the intensification of therapy with chemotherapy in combination with RT for locally advanced disease presentations. Parallel to the changing landscape of combined modality therapy in the management of NPC, there has been a rapidly changing landscape of technical RT planning, treatment delivery and treatment verification. Intensity-modulated radiation therapy (IMRT) in combination with image-guided radiation therapy (IGRT) strategies offer the potential for increasing accuracy of RT and limiting radiation dose to normal tissues, thereby, increasing the probability of cure and optimal quality of life. This chapter will review the principles of radiation planning as they apply to advanced radiation therapeutic strategies, the fundamentals of IMRT and IGRT and the emerging body of data demonstrating excellent results from IMRT. As well, the potential of IGRT in the management of NPC will be discussed. With the expectations of excellent loco-regional control, future efforts must be directed toward limiting RT-related toxicity. Despite excellent loco-regional control, some patients will still succumb to distant metastases. Evolving systemic strategies are being undertaken to reduce the probability of developing metastases. Combined modality therapy may cause more side effects. These efforts highlight the importance of reducing RT side effects. While RT can be used to re-treat patients with recurrent disease and palliate symptoms in incurable patients, this chapter will focus on the initial curative management of NPC.

Nasopharyngeal Carcinoma: Keys for Translational Medicine and Biology,
edited by Pierre Busson.

INTRODUCTION

Radiation therapy is the primary curative treatment modality for patient with NPC. For head and neck (H&N) radiation oncologists, RT planning for NPC is the most challenging H&N subsite due to the complexity of skull base anatomy and narrow safety margins due to near-by critical organs such as the cochlea, brainstem, brain, optic chiasm, spinal cord and mandible. However, a radiation oncologist can treat wider normal tissue margins than are accessible to the surgical oncologist as the near-by normal structure may tolerate radiation doses close to the prophylactic radiation dose required to sterilize microscopic disease. The nasopharynx is closely bounded by complex normal structures including the skull base superiorly, infratemporal fossa laterally and neurovascular bundle postero-laterally. Cancers of the nasopharynx have the propensity to invade these critical normal tissue regions which render the disease surgically unresectable. Even without invasion of these nearby structures, surgery is technically challenging and it is often not possible to obtain wide surgical normal tissue margins needed to ensure adequacy of resection and to minimize local (nasopharynx) recurrence. NPC surgery should only be undertaken in specialized centers of surgical expertise and excellence. Surgery dose have a role in the post-RT management of the neck. Patients should be considered for surgical salvage of regional (nodal) RT failure. The same surgical principles of salvage neck dissection for any H&N mucosal cancer can be applied to NPC. The incidence of isolated neck recurrences following RT is low in NPC. Hence, ≤5% of patients will be eligible for a neck dissection.[1-3] The local and regional control is excellent with single modality radiation therapy for early stage, nonbulky disease. Combined modality therapy with RT and chemotherapy for patients with locally advanced disease has been a major advancement in the management of NPC. The evolving role of chemotherapy and molecular targeted agents is discussed in Chapter 10 by Hui and Chan. Intensity-modulated radiation therapy (IMRT) is a form of 3-dimensional (3D) conformal radiation therapy. H&N RT targets are complex 3D volumes. The potential benefit of IMRT is the ability to plan and deliver highly conformal radiation that encompass H&N RT targets while limiting dose to nearby critical structures. Imaging a patient during a course of RT to ensure that the patient (and tumor) is in the same position as the RT plan is referred to as image guided-radiation therapy (IGRT). The ability to identify treatment set-up errors enables the implementation of corrective strategies for treatment set-up displacements or errors. IGRT offers the potential for increasing the accuracy of radiation treatments and potentially reducing late normal tissue injury by enabling the reduction of uncertainty planning margins (see PTV below) inherent in all RT plans. These uncertainty margins are in fact *normal tissue* margins. Large uncertainty margins may be a contributing factor to some radiation side effects. IGRT offers the potential to adapt to patient-specific changes during a course of therapy. The potential benefit is that treatment can be tailored to the individual instead of applying population-based treatment strategies.

Early clinical results with IMRT (with or without chemotherapy) have shown excellent loco-regional control. Unfortunately, approximately 20-30% of patients will still develop incurable metastases leading some people to view NPC as a systemic disease. RT is loco-regional treatment. If IMRT results consistently show excellent loco-regional control then future research efforts should be directed at limiting toxicity from RT which can be a significant cause of patient morbidity after several years and even decades of cure.

Tomotherapy[4] and Intensity Modulated Arc therapy (IMAT)[4,5] are forms of IMRT. There is limited clinical data in treating NPC with these two technologies. While there may be some practical differences between IMRT techniques related to technology-specific, planning software-specific and vendor-specific factors, this chapter will deal with the guiding principles of IMRT and a review of the clinical outcomes will be presented in context of other RT modalities. There are no clinical trials data to advocate for the use of any one specific IMRT technology.

PRINCIPLES OF RADIATION THERAPY PLANNING

The principles of radiation oncology planning have not changed with the implementation of advanced RT technologies. The radiation oncologist must apply the established oncologic and radiobiological principles in the conformal RT era. As newer technologies replace what is now state-of-the art RT, the fundamental principles will continue to apply. For example, the International Commission on Radiation Units and Measurements (ICRU) provides standards and guidelines for radiation target definition as well as planning and dose prescription. However, the principles of ICRU remain important in the conformal RT era and they are not specific to any technology.[6,7] These documents contain specific language that highlights this very important issue, "It must be stressed that the prescriptions of GTV(s) and CTV(s) are based on general oncologic principles and they are independent of any therapeutic approach…. Their definition must *precede* the selection of treatment modality and subsequent planning procedures."[6,7] CTV and GTV are defined below. It is relevant to review the principles of RT planning as they apply to NPC management. A new ICRU document (ICRU 83) specifically addresses IMRT but the RT target volume definitions have not changed.

Treatment Preparation and Planning

The preparation of a patient for RT requires a number of assessments and baseline investigations that are important to the long-term health of NPC patients. In addition to imaging staging tests, a thorough history and clinical examination is critical. A complete clinical assessment includes a direct flexible fiber-optic naso-laryngoscopic examination of the nasopharynx and complete evaluation of surrounding mucosal surfaces. A clinical assessment of the cranial nerves should be done in all patients. Clinical examination can provide invaluable information for tumor target localization during the planning process as disease can be directly visualized that may not be seen by modern imaging techniques. Patients should undergo pretherapy dental evaluation and counseling. Baseline audiometry and ocular evaluation is recommended.

All patients should undergo a specialized planning computerized tomography (CT) scan with appropriate H&N immobilization usually consisting of a mask and head rest.[8] Magnetic resonance (MR) scan registration and fusion techniques facilitate gross disease delineation particularly when tumors are near or involve the skull base (Fig. 1A-C).[9] Positron Emission Tomography (18FDG-PET) can also provide important planning information (Fig. 1D).[10] Currently, imaging registration and fusion technologies used in the planning process are based on 'rigid' modeling which can not account for all patient deformation and rotation discrepancies between the primary

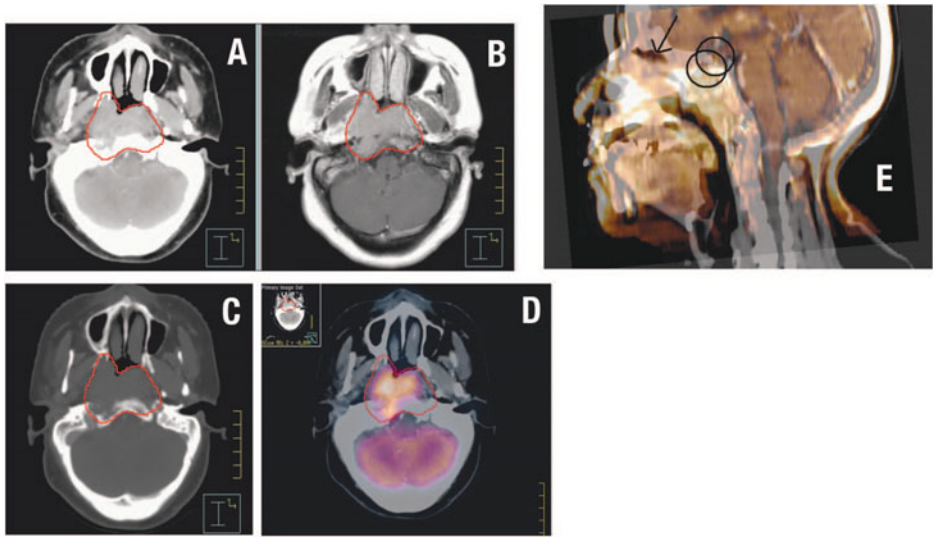

Figure 1. MR and PET Registration/Fusion with Planning CT scan. NPC is contoured. A) Planning CT scan; B) MR registered and fused to planning CT scan; C) Planning CT scan, 'bone' window setting; D) Planning CT scan registered and fused to PET scan; E) Registration/Fusion mismatch error intentionally created to demonstrate inaccuracy in overlay of optic chiasm (arrow points to displacement of chiasm within the circle regions).

planning scan and the secondary registration image modality e.g., MR scan. For a more detailed review of registration strategies, the reader is referred to references 11 and 12.[11,12] Care and caution must be taken when using secondary images to assist contouring of gross disease and critical normal organs. For example, any rotational mismatches between the fused planning CT and MR scans can adversely affect the accurate contouring of the optic chiasm as this anatomical region is very sensitive to these rotations (Fig. 1E).

Radiation Dose and Fractionation Schedule

A standard radiation total dose range for gross disease for mucosal H&N cancers is 66-70 Gy using a standard dose per fraction 1.8-2 Gy. This total and fractional radiation dose is relevant for NPC RT. Some centres routinely use an additional 'boost' to the nasopharynx. A radiation 'boost' is typically the final phase of radiation therapy used to only treat gross disease. A boost can be used after prophylactic RT has been delivered to regions at-risk for microscopic involvement or as a dose-escalation strategy. In Hong Kong, 'standard' dose-fractionation schedules for NPC were previously influenced by serious radiation therapy treatment unit shortages and large fractional doses, 3.8-4.2 Gy, were used until the early 1980s to minimize the total number of treatments.[13] In a NPC population-based model of 1008 early stage (Ho classification) patients treated between 1976-1985 with large fractional doses, Lee and colleagues examined possible radiobiological parameters predictive of local control and treatment toxicity. In this study, fraction size did not impact on local control.[14] She demonstrated an association

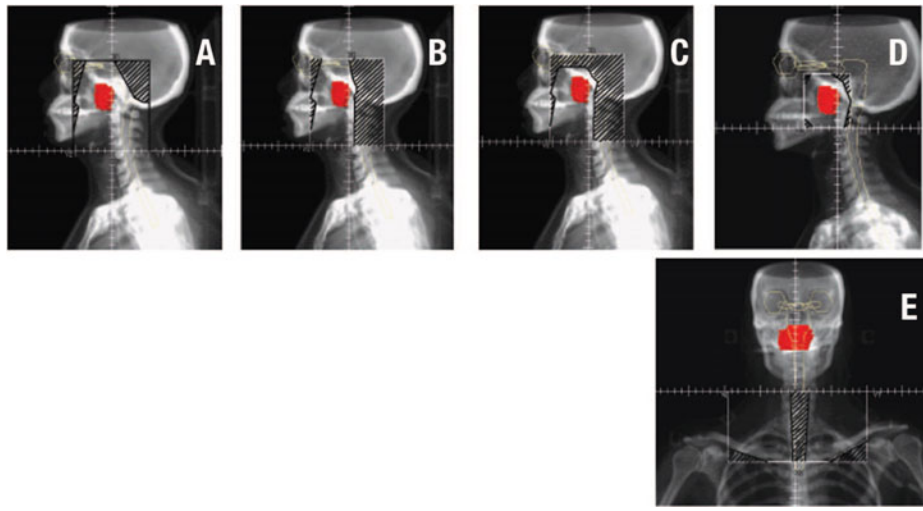

Figure 2. Example of Multi-phase 2DRT. NPC is shown. A) Phase 1 Lateral fields; B) Phase 2 Lateral fields with spinal cord shielding; C) Phase 3 Lateral fields with optic chiasm and optic nerve shielding; D) Nasopharynx boost lateral fields; E) Anterior low neck field with midline spinal cord and lung shields.

between larger fraction size, (3.5 Gy) and shorter (accelerated) overall treatment time with the development of symptomatic temporal lobe necrosis. This observation is consistent with the radiobiological principle that there may be increased late normal tissue damage when larger dose per fraction are delivered.[15,16]

The delivery of non-standard radiation dose per fraction or the use of non-standard RT schedules is termed altered fractionation. There is limited retrospective[17] and prospective[18] clinical data showing improved cancer control using altered fractionation and unacceptable normal tissue injury can result from the use of these regimens.[19] Therefore, altered fractionated schedules can not be recommended as standard of care for NPC patients.

Recently, technology driven factors have influenced dose-fractionation scheduling as well as radiobiological principles. 2-Dimensional RT (2DRT) requires multiple phases of treatment. Typically, an initial large radiation portal is used to encompass gross disease and routes of microscopic tumor spread. This initial phase can not be continued to a dose required to sterilize disease due to spinal cord dose constraints. A second phase is used to limit dose to the spinal cord. Following a so-called "microscopic" dose, subsequent phase(s) treat sites of gross disease only (Fig. 2). With IMRT, optimal dose conformation and tissue sparing may be better achieved with single phase therapy. Examples of this approach have been described as simultaneous integrated boost (SIB)[20] and simultaneous modulated accelerated radiation therapy (SMART) boost.[21] This strategy has also been commonly described as "dose painting". The key principle of these strategies is that the higher gross disease dose and lower microscopic dose must be delivered in one plan over the *same number of fractions*. This had lead to the emergence of non-standard fractional doses (<1.8 Gy or >2 Gy) (Fig. 3). Clinical trials are now investigating these dose-fractionation schedules.

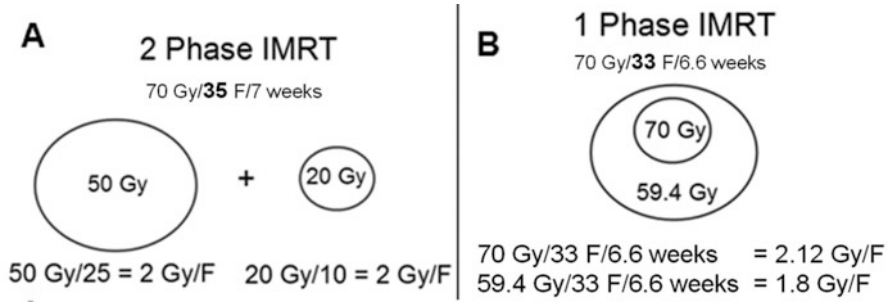

Figure 3. A comparison of two-phase IMRT dose-fractionation to an example of a one-phase IMRT dose-fractionation. A) 2-phase IMRT plan using 'standard' 2 Gy fractions (F) throughout treatment. B) 1-phase IMRT plan treated over 33 fractions. Prophylactic (microscopic) dose kept to 'standard' 1.8 Gy per fraction. Hence, gross disease is treated 2.12 Gy per fraction to desired total dose of 70 Gy.

Principles of ICRU 50/62

ICRU Report 50: Prescribing, Recording and Reporting Photon Beam Therapy was published in 1993. The supplement to ICRU 50, ICRU Report 62: Prescribing, Recording and Reporting Photon Beam Therapy was published in 1999. These documents provide important guidance to the definition of radiation therapy targets (GTV, CTV) and associated geometric expansions that account for uncertainties that may occur during a course of RT (PTV, PRV). Gross Tumor Volume (GTV) is defined as any gross tumor determined by clinical examination and imaging. Clinical Target Volume (CTV) is a normal tissue margin, encompassing GTV that accounts for subclinical spread of cancer. It is common that multiple CTV(s) are defined for volumes to be treated to separate gross disease or prophylactic doses of radiation i.e., CTVI, CTVII. These suffixes are not consistently applied in the current literature and suffixes indicating dose in Gray (Gy) are useful i.e., CTV70, CTV50. Planning Target Volume (PTV) is a volume that accounts for all geometric uncertainties that must be accounted for to ensure adequate CTV coverage with the prescription radiation dose. These uncertainties include internal motion (e.g., swallowing) and day to day treatment set-up errors (e.g., variations in H&N mask fitting). Thus, it is the PTV that is the planning target not the CTV. Similarly to PTV, a volume surrounding a clinically defined normal Organ-at-Risk (OR) is defined as Planning Organ-at-Risk Volume (PRV) accounting for geometric uncertainties around an OR (Fig. 4).[6,7]

In NPC RT planning, it is common for PTV margins to overlap with OR(s) and PRV(s) (Fig. 4). While compromises may need to be made in the planning process when PTV is near or overlaps critical structures such as the brainstem, it must be emphasized that the uncertainties inherent in PTV remain even in a clinically acceptable appearing RT plan. There are several strategies that can be used to define a clinically relevant PTV.[22] A commonly used population-based model, 'van Herk formulation', defines random and systematic error components of PTV. This 'margin recipe' can be used to derive a clinically relevant PTV from actual patient data. This is a population-based PTV model and it is not tailored to any one patient. Implicit in any clinical application of mathematical models is the needs to understand the basic assumptions. A major

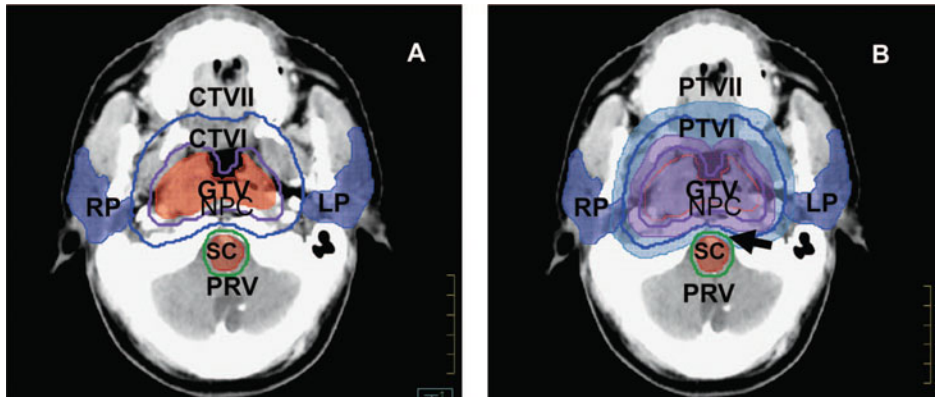

Figure 4. ICRU defined volumes. A) Primary tumor (GTV) encompassed by Clinical Target Volume to be treated to gross disease dose (CTVI) and microscopic dose (CTVII). Spinal cord (SC) is encompassed by Planning Organ at Risk Volume (PRV). Right (RP) and left (LP) parotid glands are shown as examples of Organs at Risk. B) CTVI and CTVII are expanded by 5 mm geometric margins to generate PTVI and PTVII. Note PTVII overlaps with spinal cord PRV.

assumption in the van Herk formulation is that all displacements and discrepancies of CTV during treatment are rigid displacements (superior-inferior, anterior-posterior, cranial-caudal). This model does not account for changes in patient shape (deformation) over a course of therapy.[23] It has been quantitatively shown that H&N patients are prone to deformation and rotational displacements during a course of RT.[24,25] PTV is a critical concept in RT planning and treatment delivery. Many RT centers do not have PTV margin data derived from their patient population. A 5 mm geometric PTV margin around is commonly used in practice in this setting and in some RT clinical trials.

Radiation Therapy Target Delineation

The increasing use of conformal radiation therapy techniques requires the radiation oncologist to delineate (contour, segment) many complex volumes including GTV and CTV. PTV is not a contoured volume but a geometric margin of uncertainty. The most critical information required for contouring is accurate clinical and radiologic staging. Other tumor factors such as histopathology subtype classification, does not impact the contouring process. Two challenges for the radiation oncologist include targeting of the neck and targeting of the primary.

There is limited data about the specific anatomic failure patterns after RT for NPC. Recurrences are commonly reported as local, regional and metastatic and this vernacular does not specify the anatomic regions bounding the primary site or neck in a way that is informative to the contouring process. As such, the radiation oncology discipline has adopted consensus guidelines for CTV delineation of the node-negative neck based on surgical pathologic data.[26] Guidelines for CTV contouring for node-positive disease have been proposed.[27] While practically useful, these guidelines may not reflect patterns of recurrence specific to NPC. One potential benefit of intensity-modulated radiation therapy (IMRT) is parotid sparing and avoidance of xerostomia (dry mouth).

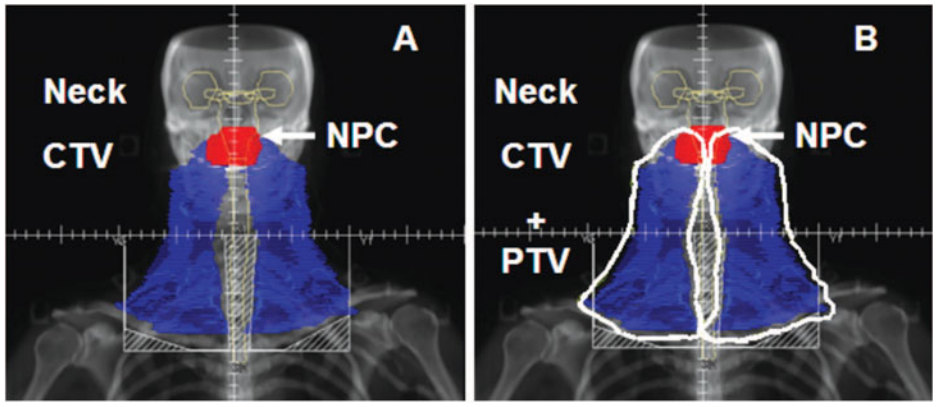

Figure 5. Conventional 2DRT fields compared to conformally delineated neck nodal targets. A) Typical lower neck anterior field does not encompass node-negative 'consensus' neck CTV (shown as shaded region). Primary tumor is shown (NPC). See reference 28 for node-negative CTV consensus. B) When a 5 mm PTV expansion is applied to CTV (PTV shown as white outline surrounding PTV), under-coverage of conformal target is more apparent.

Xerostomia is a very common consequence of prophylactic neck RT in patients treated with nonconformal techniques. Cannon et al reported parotid gland recurrences in NPC patients as a consequence of intentionally limiting dose to the parotid gland in an attempt to avoid xerostomia.[28] Thus in some patients, the parotid gland should be delineated as part of the nodal CTV. There is no established guideline for inclusion of the parotid in the neck nodal CTV and the challenge remains about when to include this region given the potential consequence of permanent xerostomia and increased risk of osteoradionecrosis of the mandible.

The use of the consensus guidelines for neck CTV delineation has resulted in a change in the neck CTV and PTV coverage compared to 2DRT (Fig. 5).[29] We reported less than 2% failure close to a midline spinal cord shield in the low neck that is typically used in 2DRT. [30] This spinal cord shield routinely shielded the medial PTV when applied to the consensus neck CTV. This issue highlights again the need for detailed anatomically-specific patterns of failure following conformal RT for NPC.

GTV delineation is a difficult clinical task and we have shown inter-observer variability among 6 experienced H&N radiation oncologists and 2 neuroradiologists when contouring GTV on contrast enhanced CT scan, noncontrast CT scan and PET-CT scans from patients with oropahrynx cancer.[31] Given that nasophraynx pimary site delineation is potentially a more complex task, it is possible that inter-observer variations in GTV and CTV delineation could be one determinant of loco-regional control and normal tissue toxicity.

Similar general principles are applied to CTV delineation across the world. It is generally accepted that CTV (to be treated to a prophylactic dose) must include gross disease and routes of subclinical spread with particular attention to the skull base and comprehensive neck nodal RT.[32-36] Seemingly subtle differences in CTV contouring may have significant normal tissue consequences. Table 1 lists the commonly accepted regions for CTV delineation as well as highlights some of the areas of uncertainty and possible normal tissue consequences of over-inclusion of normal tissues in the CTV.

Table 1. Clinical target volume in NPC

Clinical Target Volume (CTV)	Controversy/Uncertainty About Extent of Coverage	Potential Normal Tissue Consequence
Primary		
Nasopharynx		
Clivus	What extent of clivus should be included? Should 1/2-1/3 be included if clivus uninvolved and the entire clivus if involved or T3, T4?	brainstem
Skull base		
– *foramen lacerum*		
– *foramen ovale*		
– *foramen rotundum*		
– *petrous portion of temporal bone (including carotid canal)*	Should the carotid canals be excluded for T1, T2* or when primary involves contralateral nasopharynx?	brain, cochlea, middle ear
Sphenoid sinus	Should just the inferior sphenoid be included for T1, T2* disease or when uninvolved? Should the entire sphenoid if involved or T3,T4?	brain, optic chiasm
Cavernous sinus	Can the cavernous sinus be excluded for T1, T2*?	brain, brainstem, optic chiasm
Ethmoid sinus	Should the posterior 1/3 of bilateral ethmoid sinuses be included in all cases?	optic nerve, eye (retina), orbital muscles
Oropharynx (at least 1 cm uninvolved mucosal margin on primary disease)		
Nasal cavity (at least 1 cm uninvolved mucosal margin on primary disease)	Should the posterior 1/4-1/3 of bilateral nasal cavity be included for all cases?	nasal cavity mucosa, nasal hair follicles
Maxillary antrum	Should the posterior 1/4-1/3 of bilateral maxillary antrums be included for all cases?	muscles of mastication, nasal cavity mucosa, nasal hair follicles
Pterygoid fossae		
Parapharyngeal space		
Infratemporal fossa	What extent of infratemporal fossa coverage is required for early disease?	mandible, muscles of mastication
Lymph Node Region		
Retropharyngeal nodes	Should this nodal group be delineated inferiorly to the hyoid or caudal aspect of C2?	pharyngeal constrictor muscles
Level 2-5		
Level 1B	Can level 1B excluded in N0 cases or when level 2a is not involved?	mandible, submandibular gland, floor of mouth mucosa

*T2 category includes T2a, T2b

Radiation Therapy Quality

One of the criticisms of the landmark NPC Intergroup trial 0099 that demonstrated improved disease-free survival (DFS) and overall-survival (OS) in NPC pateints using concurrent and adjuvant cisplatin-based chemotherapy was the unexpectedly low 5-year DFS, 29% and OS, 37%, in the standard RT alone arm.[1,37] Several explanations have been suggested including the relatively high incidence of WHO Type 1 NPC (28%) in this North American study population. The 5-year DFS and OS of patients treated at the Princess Margaret Hospital (PMH) during a similar time period was 62% and 48% which was higher than the RT alone arm of Intergroup 0099 but lower than the combined chemotherapy and RT study arm. Single institutional experiences can not be directly compared to the results of a randomized trial. It is, however, noteworthy that the PMH results were in line with other major centers during this time period.[3] One potential confounding factor may have been that patients were treated with 2DRT without the routine use of a planning CT scan. However, no conclusions can be made about the quality of RT planning and delivery in the Intergroup 0099 trial as there was no centralized RT quality assurance review. The limitation of 2DRT planning without the use of a planning CT scan was demonstrated in patients treated at PMH during an overlapping era with Intergroup 0099.[8] There may be several medical advances over a period of years that could lead to improved patient loco-regional control, DFS and OS including advances in detection, diagnosis, staging, systemic therapy and advancements in RT planning/delivery. Several authors have reported improved RT loco-regional control outcomes when compared to prior institutional treatment time periods and RT technical advances may have played a role.[13,38,39] Excellent loco-regional control has been reported with early intensity-modulated radiation therapy (IMRT) experience as an example of advanced RT (Table 2). In a phase III trial of non-nasopharynx locally advanced H&N squamous cell carcinomas conducted by the Trans- Tasman Radiation Oncology Group (TROG) investigating standard concurrent chemoradiation with or without tirapazemine, all RT plans were subjected to expert peer review. Twenty-percent of patients were found to have major protocol deviations in the radiotherapy plan. These protocol deviations were, for the first time, associated with increased risk of death (HR = 1.56; $p \leq 0.0001$), any failure (HR = 1.65; $p < 0.0001$) and loco-regional failure (HR = 1.82; $p = 0.0002$).[40] Taken together, these data support that radiation quality is an important factor in determining outcome for NPC patients. Quality control is an important aspect of any RT department and quality assurance review should be a standard practice in all RT NPC trials.

Radiation Therapy Treatment Strategies

2-Dimensional Radiation Therapy

The principles of 2DRT planning have been briefly discussed above. 2DRT planning is field-based with field placement usually using boney and sometimes soft-tissue surrogate for tumor unless a planning CT scan is used.[41] Retrospective series have shown excellent local and regional control with modern 2DRT with 5-year local control and regional control ranges 81-84% and 80-94%, respectively. In these series, 20-62% of the patients received chemotherapy.[2,42,43] To date, all RT trials evaluating the role of chemotherapy with RT have been done with 2DRT. Table 2 lists the results from randomized trials evaluating the role of concurrent chemotherapy with RT if local, regional or loco-regional control was reported.[1,37,44-46]

Table 2. Clinical results of 2DRT and IMRT in NPC

Institution	Year of Publication	Patient Number	Chemo-therapy	RT Technique	Total Dose	Reported Outcome Period	Local Control	Regional Control	Loco-regional Control	Metastases-Free Survival	Overall Survival	Ref.
Phase III concurrent chemoradiation trial												
Intergroup 0099#	1998, 2001	147	53%	2DRT	70 Gy Primary 66-70 Gy Nodes	3-year RT vs CRT	67% vs 92 %*	86% vs 91%*		65% vs 87%*	5-year 37% vs 67% (<0.001)	1,37
Taichung Veterans General Hospital	2003	284	50%	2DRT	70-74 Gy	5-year RT vs CRT	72.6% vs 89.3% (p = 0.0009)	92.% vs 96.8% (n.s.)		69.9% vs 78.7% (n.s.)	54.2 vs 72.3% (P = 0.0022)	45
Queen Mary Hospital#	2004	219	50% CRT 100% adjuvant chemo	2DRT +/– boost	66-68 Gy +/– boost	3-year RT vs CRT			72.4% vs 80% (n.s.)	30.6% vs 8.2% (p = 0.026)	76.8% v.s. 86.5% (p = 0.026)	46
Hong Kong Nasopharyngeal Cancer Group (NPC-9901)#	2006	348	49%	2DRT +/– boost	68 Gy +/– boost	3-year RT vs CRT	89.2% vs 95.3%*	92% vs 96.5%*	82% vs 92% (p = 0.005)	73% vs 76% (n.s.)	78% vs 78% (n.s.)	44
IMRT												
Non-randomized												
University of California-San Francisco	2004	118	90%	IMRT +/– brachytherapy boost	70 Gy +/– boost	4-year	96%	98%		72%	74%	33, 57, 58, 59
Queen Mary Hospital	2004	33 (early disease)		IMRT	68-70 Gy	3-year	100%	92.3%		100%	100%	34
Prince of Wales Hospital	2004	63	30%	IMRT+/– brachy-theapy boost	66 Gy +/– boost	3-year	92%	98%		79%	90%	60

continued on next page

Table 2. Continued

Institution	Year of Publication	Patient Number	Chemo-therapy	RT Technique	Total Dose	Reported Outcome Period	Local Control	Regional Control	Loco-regional Control	Metastases-Free Survival	Overall Survival	Ref.
Non-randomized (continued)												
Queen Mary Hospital	2006	50 (locally advanced)	68%	IMRT	76 Gy	2-year			95.7%	94.2%	92.1%	21
Memorial Sloan-Kettering Cancer Center	2006	74	93%	IMRT	70 Gy Accelerated RT	3-year	91%	93%		78%	83%	61
Multi-center, China	2009	323	94%	IMRT	66-69.75 Gy	3-year	95%	98%		90%	90%	36
Phase II trial												
RTOG	2008	68	84%	IMRT	70 Gy	2-year (estimated)	92.3%	90.5%	90.5%	85.7%	79.1%	62
Phase III trial												
Queen Mary Hospital#	2008	82 (40 vs 42)		2DRT +/– boost vs IMRT	68 Gy +/– boost vs 70 Gy	4-year	71.7% vs 90.5% (p = 0.019)	100% vs 92.9 (n.s.)		90% vs 81.7% (n.s.)	91.7% vs 85% (n.s.)	63

#Study terminated early 2DRT—2-dimensional radiation therapy; IMRT—intensity-modulated radiation therapy; RT—radiation therapy alone arm; CRT—chemoradiation arm; *crude rate; n.s.—not significant.

Conformal Radiation Therapy

In distinction to 2DRT, a planning CT scan must be performed for *3-dimensional conformal radiation therapy* (3DCRT) planning. Planning is not field-based but 3D volume-based. RT planning decisions are made about radiation beam geometry, weighting and modifiers ('forward' planning). Simply put, radiation field parameters are still manipulated. However, the uniformity of a radiation beam intensity (fluence) across a beam from the treatment machine is not manipulated within the treatment unit head.

Intensity-modulated radiation therapy (IMRT) is similar to 3DCRT in that the planning and plan evaluation is volumetric. Target volumes and OR(s) doses are evaluated using dose-volume histograms. However, 'inverse' planning processes are applied in which dose-volume and dose priority objectives are stipulated at the beginning of the planning process and then computerized 'optimization' is done to meet the dose volume objectives.[47-49] It should be emphasized that the dose volume objectives should be based on clinical data where available for tumor control and normal tissue tolerances. Multi-leaf collimators within the treatment unit head modulate the intensity of the radiation beam within the treatment unit head and the fluence across a beam is non-uniform (Fig. 6). A detailed discussion about the technical aspect of IMRT is beyond the scope of this chapter and

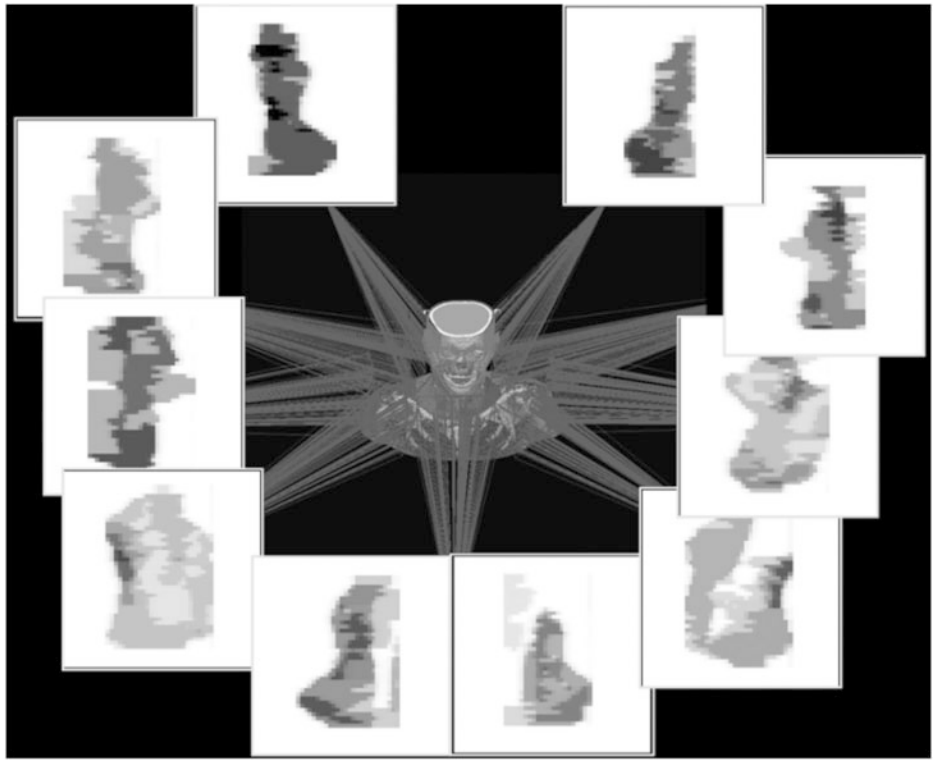

Figure 6. Nine field IMRT beam arrangement and fluence maps. Beam fluence (fluence map) is shown for each beam. The heterogeneity of each beam fluence map demonstrates the non-uniformity of beam intensity for IMRT-generated plans.

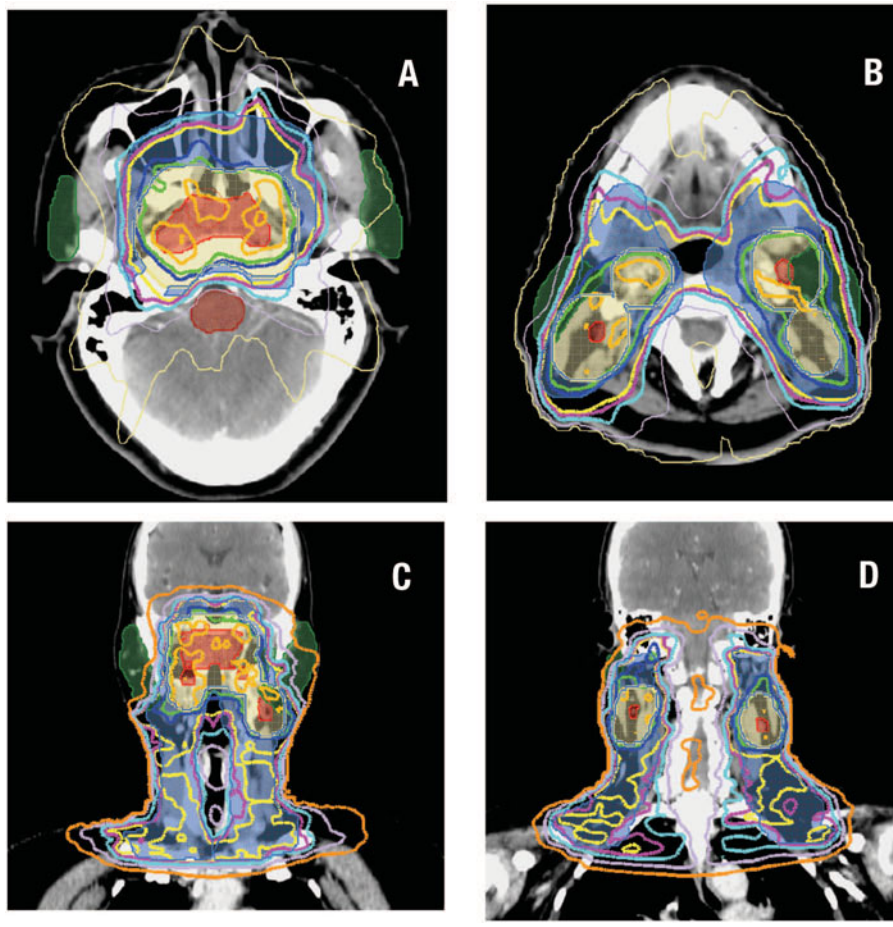

Figure 7. One-phase IMRT plan. Dose prescription are 70 Gy/35 fractions to gross disease PTV and 56 Gy/35 fractions to microscopic dose PTV. A) Axial dose distribution through primary; B) Axial dose distribution through upper neck; C,D) coronal dose distributions. Dose distributions demonstrate conformality of IMRT plans. Inner to outer shaded areas—GTV, PTV70 (to be treated to 70 Gy), PTV56 (to be treated to 56 Gy). Outer to inner lines—30Gy, 45 Gy, 53.2 Gy, 56 Gy, 58 Gy, 66.5 Gy, 70 Gy, 73.5 Gy isodose lines.

the reader is referred to reference 50.[50]A major success of conformal radiation therapies is the ability to create concave dose distributions (Fig. 7),[51] improve conformality of target coverage and create step dose gradients between target and normal tissue (Fig. 7). From the radiation oncology perspective, target delineation, uncertainty assessments, plan evaluation and treatment verification principles are the same for 3DCRT and IMRT.

There are no randomized studies comparing 3DCRT to IMRT. Several authors have shown that IMRT provides more conformal target coverage and normal tissue sparing[52-54] For early stage NPC, there may not be clinically apparent differences between IMRT and 3DCRT. In more advanced NPC, Hunt and colleagues from Memorial Sloan-Kettering Cancer Center (MSK) compared 2DRT, 3DCRT and IMRT planning for NPC. Twenty-three

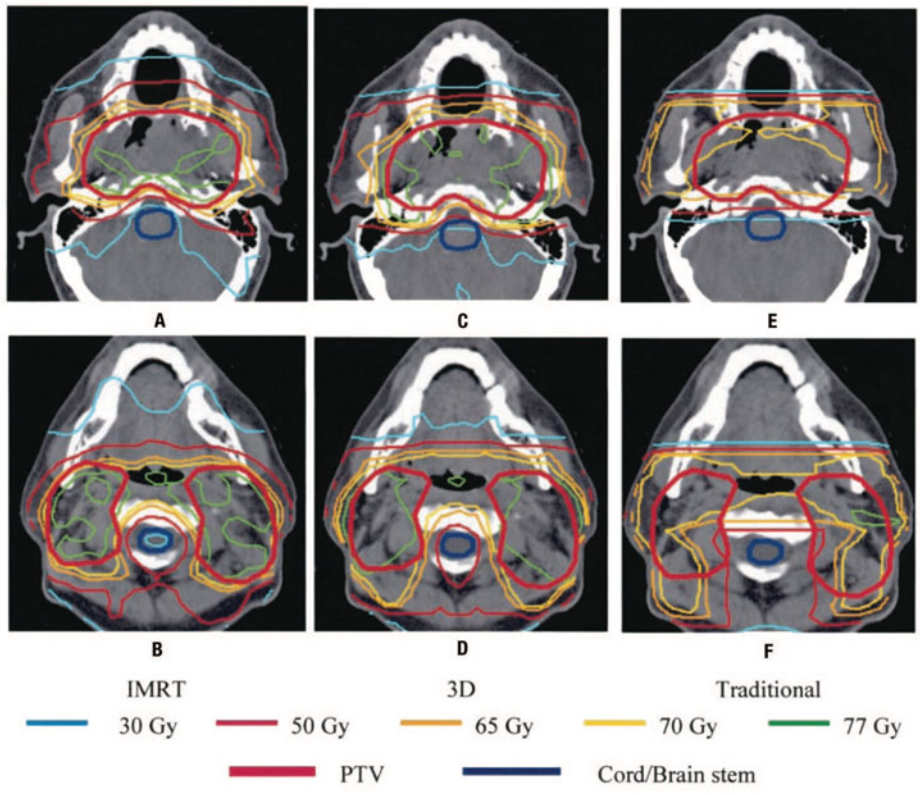

Figure 8. Axial dose distributions through nasopharynx and neck. IMRT (A,B) plan resulted in lower spinal cord maximum dose and better parapharyngeal, skull base, medial nodal coverage compared to 3DCRT (C,D) and 2DRT (E,F). Reprinted with permission from: Hunt MA, Zelefsky MJ, Wolden S, et al. Treatment planning and delivery of intensity-modulated radiation therapy for primary nasopharynx cancer. International Journal of Radiation Oncology, Biology, Physics. 2001; 49(3):623-632. Copyright 2009 Elsevier.

patients were treated clinically with IMRT and a parallel planning study was performed for 2DRT and 3DCRT (Fig. 8). Hunt et al was able to achieve a lower spinal cord dose with IMRT compared to 3DCRT, 2DRT with maximum cord doses of 49 Gy, 44 Gy and 34.5 Gy, respectively. IMRT provided better target coverage in parapharyngeal region, skull base and medial nodal regions as well as lower dose to all normal tissues.[54] These authors and others have reported difficulty achieving parotid sparing dose-volume constraints. Unlike other H&N mucosal subsites, the retropharyngeal nodal region and level 2b must be prophylactically treated in all patients and the PTV volume surrounding this CTV will always overlap the parotid gland.[55,56] It is unlikely that a randomized trial comparing IMRT to 3DCRT will be possible as many centers will not have equal experience with both therapies.

In a seminal series of reports, University of California-San Francisco (UCSF) investigators reported their IMRT single institution experience.[32,57-59] In their last report, 118 patients with Stage I-IV NPC (AJCC 1997) were included. The total IMRT dose was

70 Gy/33 fractions but 22% of patients received a brachythrapy boost. Ninety percent of patients received chemotherapy. The median follow-up was 2.5 years. The estimated 4-year local and regional control was 96% and 98%, respectively. Unfortunately, 28% of patients developed metastases and the OS was 74%. Skull base necrosis and temporal lobe necrosis was observed.[57] In an earlier report 5/67 patients experienced Grade 5 hearing loss but 29/67 presented with T3/4 disease and comprehensive skull base radiotherapy was likely required.[59] Lin et al reported similar excellent loco-regional control is a recent large series of 323 patients.[36] Table 2 lists recent reported series demonstrating excellent loco-regional control with IMRT.[32-34,36,57-61]

The Radiation Therapy Oncology Group (RTOG) conducted a Phase II study to evaluate the generalizability of the UCSF results. A preliminary report of RTOG 0522 showed an estimated 2-year local and regional control for 68 patients accrued to this trial was 92.3% and 90.5%, respectively with a median follow-up of 2 years. Distant metastases-free survival was 85.7%.[62] It should be noted that all participating centers had to obtain centralized IMRT quality assurance accreditation before being allowed to accrue patients to this study. A small randomized phase III from Queen Mary Hospital comparing IMRT to 2DRT in early stage NPC was terminated early because of marked differences in local control favoring IMRT. Eighty-two patients were enrolled in this trial before trial closure. The 4-year local control was 90.5% vs 71% ($p = 0.019$) for IMRT and 2DRT, respectively after a median follow-up of 4 years (Table 2).[63]

The excellent loco-regional control with IMRT even in advanced stage disease to date is extremely promising and the emerging data demonstrates reproducibility of results in major centers with experience with NPC. These data support the routine use of IMRT to treat NPC patients even in the absence of a large randomized trial.

IMAGE-GUIDED RADIATION THERAPY (IGRT)

Image-guided radiation therapy (IGRT) refers to patient imaging acquired during a course of radiation therapy to verify the patient position during the treatment. Historically, all radiation treatments have incorporated some form of image guidance. The most rudimentary IGRT is the well established practice of acquiring 2D 'beam's eye' views of radiation treatment fields. The imaging format is usually a mega-voltage (MV) image acquired just prior to treatment or during treatment. These images may be in hardcopy or electronic portal imaging (EPIDs) formats. Typically, the radiation oncologist will review the image after the patient has been treated and continue therapy with or without a set-up error or 'displacement' correction. This strategy is referred to as 'off-line'. 'On-line' strategies require a verification image assessment at the treatment unit where set-up correction can be performed prior to therapy. More advanced strategies employ frequent verifications throughout a course of treatment and daily IGRT has been implemented by some centers.

The major advances in IGRT have been the development of technologies that enable acquisition of full 3D verification images 'in the treatment room'. A detailed review of 'in room' IGRT technologies is beyond the scope of this text and readers are refereed to reference 24. 'In room' technologies all employ either MV or kilo-voltage (kV) CT imaging. More recently, 'in the treatment unit' technologies have been developed. kV cone-beam CT scan (CBCT) imaging is an example of this technology in which a CBCT unit is incorporated into the treatment unit.[64-66] Using CBCT, volumetric or

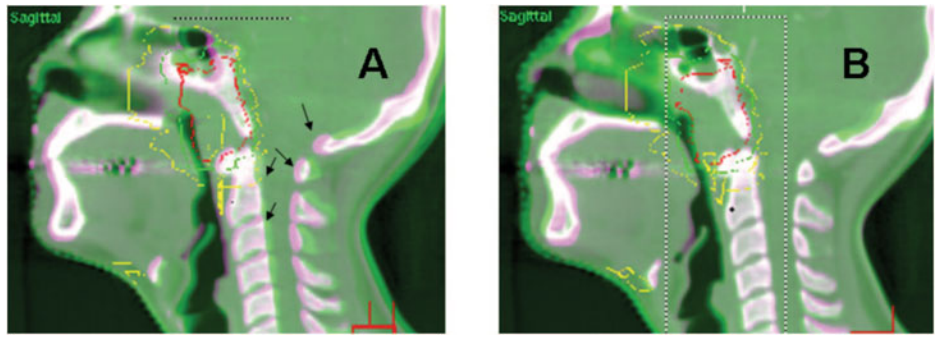

Figure 9. Three-dimensional (3D) image-guided radiation therapy (IGRT). A) Overlay of planning CT scan (reference scan) and 3D image acquired using a cone-beam CT scan (IGRT scan) at the treatment unit. Only a representative sagittal plane is shown. Double image (highlighted by arrows) is seen between the reference scan and the IGRT scan due to set-up errors (displacements) that were detected prior to treatment. It is important to note that axial and coronal planes are also captured of a 3D image and displacement corrections are made in 3D. B) Double image is not apparent as the displacements were corrected for prior to treatment by applying treatment couch shifts to offset displacements. Note that the rectangle defines a region of interest (ROI) for image-guided analyses of displacements and corrections.

3D image datasets can be acquired during a course of RT. Image matching protocols can be developed to match internal surrogates of the target volume such as bone (bone-matching). Tumor-matching or normal-tissue matching i.e., soft tissue matching can also be performed. Patient volumetric data is usually acquired in a region of interest (ROI). For NPC patients, the skull base should be included in the ROI (Fig. 9). Skull base bone matching is a good surrogate for nasopharynx tumors which do not move day to day relative to bone. The clivus is a useful surrogate for the brainstem.

Concern has been raised about the additional patient radiation dose from the acquisition of IGRT images and the potential for second cancers.[67,68] For CBCT, this dose depends on many factors specific to the image matching technology and imaging acquisition parameters. Phantoms studies performed at PMH recorded single scan doses in the rage of 1.6-2.3 cGy for institutional scan protocols.[69] Others have reported higher doses.[67,68] Additional radiation dose can be partially accounted for by including the total imaging dose in the dose calculations if there is a clinical concern about the additional dose.

One potential benefit of IGRT is to ensure that the radiation treatment doses are reflective of the actual treatment plan delivered each day. These daily set-up variations can result in delivered radiation doses that do not accurately reproduce the original plan resulting in potential underdosing of the tumor.[70] Moreover, the delivered dose to normal structures can be unexpectedly high. Han et al reported increased parotid gland and spinal cord dose if daily IGRT was not used for conformal tomotherapy.[71] Similar results were reported by others.[72] As discussed previously, PTV uncertainties are made up of systematic and random errors. Off-line correction strategies decrease systematic errors only. Whereas, on-line corrective strategies will reduce both systematic and random errors.[73] The potential benefit of daily online corrective strategies is that PTV margins can be reduced if both systematic and random error components are reduced. PTV margins are normal tissue margins. By reducing PTV margins, normal tissue toxicity could potentially be reduced.[74]

ADAPTIVE RADIATION THERAPY

The changing view of a course of RT has evolved from being regarded as a static process to one that is dynamic (Fig. 10). Quantitative data is emerging confirming what was known to the experienced H&N oncologist, that anatomical changes during a course of radiation therapy are complex.[24,25,75] Current clinically implemented IGRT corrective strategies address rigid patient displacements well and some minor rotations can be adjusted. However, patient deformational changes are not accounted for. PTV margin recipes are derived from population data and may not be ideal for individual patients. Adaptive radiation therapy refers to adapting to individual patient changes during a course of RT. Adaptive radiation therapy was first described for non-H&N cancers but is now being investigated in H&N cancer and NPC patients.[76] Rapidly advancing technologies will enable complex replanning during treatment without delaying treatment or causing undue resource burden. These technologies include the evaluation of deformable registration technologies so that deformational changes can see and adjusted for.[77-80] Investigators

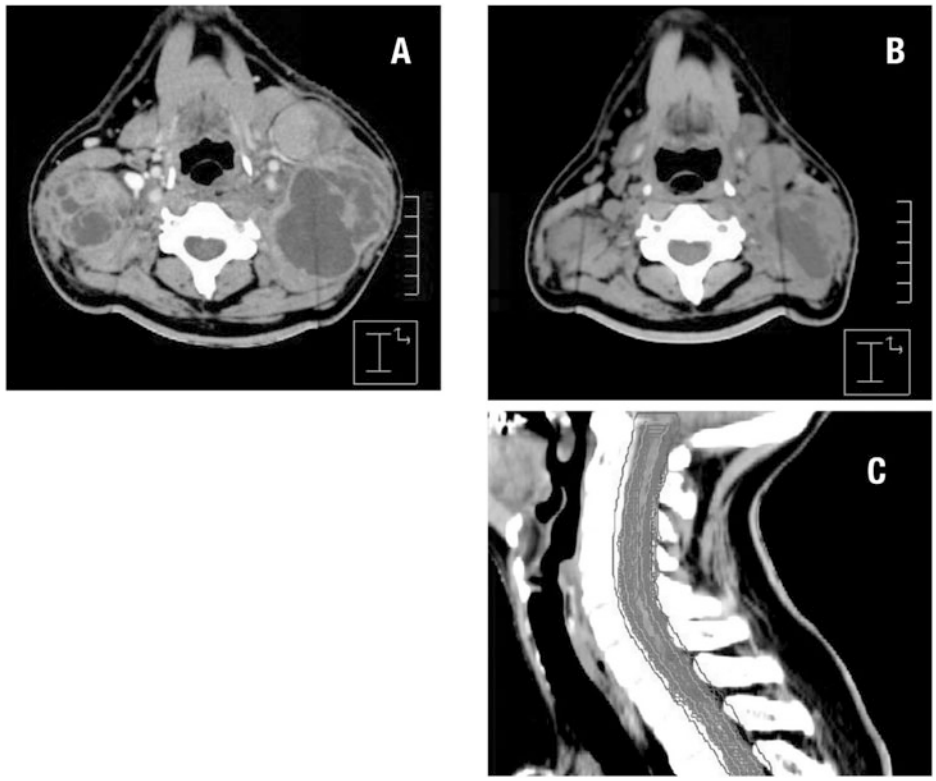

Figure 10. Examples of tumor and normal tissue changes during a course of radiation therapy illustrating that a course of radiation therapy is a dynamic process. A) Bulky bilateral neck lymphadenopathy (arrows). B) Lymph nodes have markedly shrunken by week 3 of a 7 week treatment necessitating replanning of IMRT. C) Shaded region is comprised of 35 spinal cord contours acquired from daily cone-beam CT scans over a 7 week course of IMRT. Spinal cord PRV is shown as outline. Note that shaded area is outside of PRV posteriorly demonstrating day to day spinal cord position variability.

are exploring the use of CBCT as the replanning CT scan so that additional planning CT scans do not have to be acquired.[81] In the future, radiation medicine practioners will be able to respond to treatment changes quickly. As novel molecular diagnostic and therapeutics emerge, the triggers for replanning radiation therapy may be changes in tumor microenvironment such as oxygenation.

Whether IGRT and adaptive radiation therapy will result in better outcomes is unknown. In view of the reported long-term toxicities from RT (see below), relatively small dosimetric changes may have clinical implications for normal tissues doses on the steep part of the dose response curve.

Another divergent approach to treatment changes is 'robust IMRT' modeling. Instead of replanning RT to adapt to changes, IMRT planning is modeled to account for changes such as breathing in thorax irradiation.[82] Currently, robust IMRT planning is a novel research concept.

LONG-TERM TREATMENT TOXICITY

RT for NPC can lead to serious long-term sequalae. Serious long-term toxicities have been for 2DRT and include Sensorineural hearing loss,[83-86] temporal lobe and brain necrosis,[87,88] osteoradionecrosis,[89] cranial nerve palsies,[90] optic neuropathy,[91] endocrine dysfunction,[92] carotid artery stenosis,[93] second cancers.[94] The baseline incidences of these unfortunate morbidities are unclear as some of the data comes from high dose per fraction RT or dose escalation experiences. Whether CT-based 2DRT planning, 3DCRT and IMRT can decrease the incidence of these late side effects will take years to establish. The potential to limit dose to parotid glands and decrease the probability of xerostomia is well documented.[95-101] Pow et al reported preliminary results of a small randomized clinical trial comparing IMRT and 2DRT. Better quality of life (QoL) and improved measured salivary flow at 12 months was observed in the IMRT group.[102] In a recent publication by Eisbruch et al,[103] there were no osteoradionecrosis complications in 176 patients treated with IMRT between 1996-2005 and followed for a minimum of 6 months. The tolerance limits for some organs are being better defined. In 26 patients, Eisbruch et al reported that the lowest dose delivered to the pharyngeal constrictor muscles to cause dysphagia and aspiration was 50 Gy.[104] However, some authors have reported concerns about IMRT toxicities. Rosenthal et al reported acute symptoms of headache, nausea and vomiting, scalp alopeca and oral cavity mucositis with IMRT related to the lower doses to the brainstem (>36 Gy), occipital scalp (>30 Gy) and anterior mandible (>34 Gy). Longer term concerns have been raised regarding of carotid artery complications related to higher carotid artery dose than with non IMRT techniques. The risk of second cancers has also been raised.[105-107]

CONCLUSION

To date, RT and systemic treatment strategies have been to intensify therapies. Strictly speaking, IMRT and 3DCRT can be considered *intensified* therapies as mean target doses tend to higher than with 2DRT. With improvements in target coverage, tumor margins receive higher doses than with 2DRT. Loco-regional control rates of greater than >90% have been reported by several institutions but a significant proportion of patients

will still develop metastatic disease. Patients who have undergone RT for NPC are at long-term risk of RT injury. Is it appropriate to ask, should future therapies be directed toward *de-intensification* of loco-regional therapies with *intensification* of systemic therapies? The loco-regional control of Human Papilloma virus-associated oropaharynx squamous cell carcinoma is also excellent with radiation therapy.[108] Interestingly, efforts are underway to develop clinical trials to investigate de-intensification treatment strategies in this group of patients. There are significant differences between these two patient populations including the lack of salvage surgical options for patients with locally recurrent NPC. While de-intensification is provocative, the potential consequences may be devastating if loco-regional failures increase. Dose *de-intensification* strategies should only be explored in clinical trials setting. IMRT results still need longer term confirmation. Current research strategies should include efforts to reduce radiation dose to normal tissues through improvements in conformal RT and implementation of IGRT.

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CHAPTER 10

THE EVOLVING ROLE OF SYSTEMIC THERAPY IN NASOPHARYNGEAL CARCINOMA: Current Strategies and Perspectives

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Abstract: Treatment for nasopharyngeal carcinoma (NPC) has evolved tremendously over the last decade, owing to the integration of chemotherapy into the primary radiotherapy, improvement in tumor imaging and disease monitoring, and advances in high precision radiotherapy delivery. Several randomized trials have established concurrent chemoradiation (with or without adjuvant chemotherapy) as the standard of care in advanced NPC. Current efforts are building on these earlier trials, to further test the optimal strategy of integrating neoadjuvant or adjuvant chemotherapy to further improve patient's survival and quality of life. Meanwhile investigators are developing novel and molecular targeted therapies in locoregionally advanced or metastatic NPC. This chapter will provide a basic understanding of the clinical data from randomized chemotherapy trials in NPC, current effort to integrate neoadjuvant chemotherapy to concurrent chemo-radiation in advanced NPC, and selection of high risk NPC by molecular marker of minimal residual disease for adjuvant therapy. On-going clinical studies in molecular targeted therapies in NPC, including the epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), epigenetic therapy, Epstein-Barr virus (EBV) directed immunotherapy and gene therapy, will also be discussed.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) has several peculiar characteristics compared with other head and neck squamous cell carcinoma (HNSCC) in its epidemiology, pathology,

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clinical behavior and response to treatment.[1,2] Because of its deep anatomical location, surgery is not an option as first line treatment. Therefore, all patients of newly diagnosed NPC whose disease is confined locally (nasopharynx) and regionally (neck lymph nodes), but has not spread to other parts of body (distant metastases, DM), have traditionally been treated with radiotherapy alone.

Although early stage NPC is highly curable (>90%) by radiotherapy, the cure rate for those with locoregionally advanced NPC remains unsatisfactory. Because NPC is also highly sensitive to chemotherapy, the addition of chemotherapy to radiotherapy in various combinations (delivered before, during, and after radiotherapy) has been explored to improve the cure rate. From the results of randomized clinical trials conducted in the past two decades, the addition of chemotherapy to radiotherapy has been proven to improve the treatment outcome. However, the optimal timing, dosing, duration, and regimens of chemotherapy drugs to be combined with radiotherapy remain to be defined. Despite a high response rate of NPC to systemic chemotherapy, the prognosis for patients with distant metastatic disease remains poor.[3] Moreover, NPC survivors often suffered from moderate to severe late complications, many of which result from the effect of radiation on the organs adjacent to nasopharynx and neck nodes. The use of chemotherapy in advanced cases further adds to these side effects. Therefore novel and more targeted therapies with reduced side effect need to be explored.

THE ROLE OF CHEMOTHERAPY

At the time of initial diagnosis, less than 5% of patients with NPC were found to have DM in modern series. Control of primary tumor (local control) and prevention of DM has been the major goals in NPC, whereas regional control (neck nodes) is less a problem. With the prospect of high local control rate achievable by the application of high-precision radiotherapy, DM is expected to become the predominant cause of treatment failure from NPC.[3]

Chemotherapy is the use of cytotoxic drugs to destroy cancer cells. Chemotherapy can be added to radiotherapy in order to enhance its effect on local control or to eradicate occult DM. Theoretically combination chemotherapy regimen is probably more effective in eradicating micro-metastases, although it may be practically impossible to deliver at full doses concurrently or sequentially with radiotherapy. Optimal timing of the two modalities is crucial for the success of this treatment. Although treatment strategies other than the addition of chemotherapy (three-dimensional conformal or intensity modulated radiotherapy, accelerated fractionation schedule, intracavitary boost) are currently being pursued to enhance locoregional control, systemic chemotherapy is the only available option to directly address DM. Nevertheless, it cannot be excluded that optimal locoregional control translates into a better DM control (the chicken-and-egg hypothesis, where the seeds of DM may come from uncontrolled local disease), and that a treatment primarily addressed to attack DM may also be beneficial on locoregional disease control.[4]

TIMING OF CHEMOTHERAPY AND RADIOTHERAPY

The best timing of chemotherapy given in relation to radiotherapy has been a controversial issue. Chemotherapy may be given before (neoadjuvant or induction

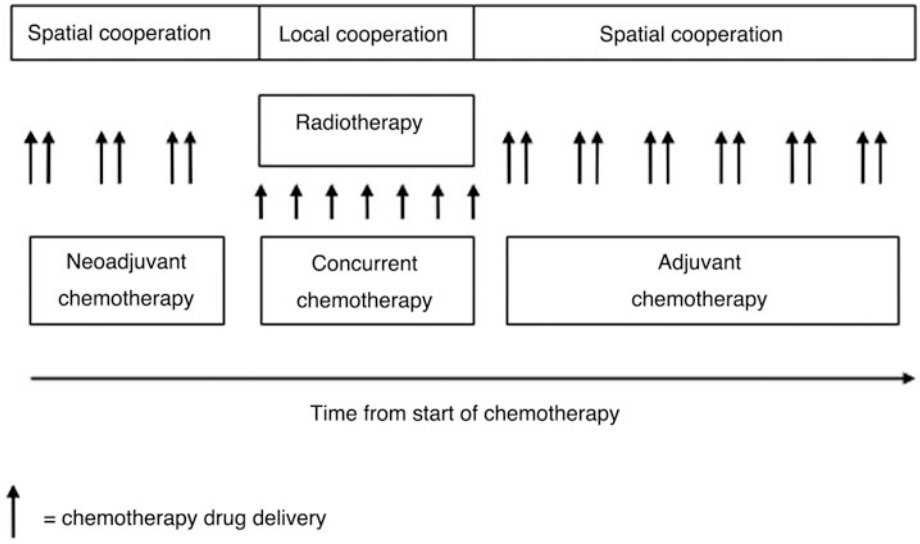

Figure 1. Scheme of integration of chemotherapy and radiotherapy.

chemotherapy), alongside (concurrent or concomitant chemoradiation), or after radiotherapy (adjuvant chemotherapy). Figure 1 illustrated the possible schemes to deliver chemotherapy in relation to primary radiotherapy.

When two treatment modalities are expected to work independently at two different targets (radiotherapy at local and regional sites, and chemotherapy on distant micro-metastases), the best therapeutic index is usually obtained when the two treatments are given at different times (i.e., neoadjuvant chemotherapy followed by radiotherapy, or radiotherapy first followed by adjuvant chemotherapy), because of the concern that interaction between the two modalities may prohibitively increase the risk of acute side effect. This strategy is called "spatial cooperation".[4] However, if local effect is the primary aim, then concurrent administration of both modalities is usually preferred. This strategy of "local cooperation" aims to produce an additive or synergistic interaction between radiation and chemotherapy (radiosensitizer). Results from head and neck squamous cell carcinomas confirm that the concurrent administration of chemoradiation is superior to sequential use in obtaining a local effect. However, acute and sometimes late reactions become the limiting factor. Drugs with non-overlapping toxicity with that of radiation are preferred.

In the past two decades, sixteen randomized controlled trials have been reported on the use of neoadjuvant, concurrent, and adjuvant chemotherapy, or a combination of these approaches in the treatment of advanced NPC (summarized in Tables 1 and 2). Available clinical data confirm the role of neoadjuvant chemotherapy in reducing DM and concurrent chemoradiation in enhancing local control. Interestingly, some locoregional effect of sequential chemotherapy and some distant effect of concurrent chemoradiation have also been observed.

Table 1. Randomized clinical trials of neoadjuvant/adjuvant chemotherapy with radiotherapy vs radiotherapy alone in locally advanced nasopharyngeal carcinoma

Institution	Year of Publication	Patient Number	Timing of Chemotherapy	DFS		OS		Benefit	Reference
				RT	CT/RT	RT	CT/RT		
Adjuvant trials									
Institute Nationale Tumori, Italy	1988	229	Adjuvant VCA × 6 cycles	4-yr: 56%	58%	4-yr: 67%	59%		14
TCOG (Taiwan Cooepative Oncology Group)	2002	157	Adjuvant PFL × 9 cycles	5-yr: 50%	54%	5-yr: 61%	55%	DM	15
Neoadjuvant trials									
PWH (Prince of Wales Hospital, Hong Kong)	1995	82	Neoadjuvant PF × 2 cycles and adjuvant PF × 4 cycles	2-yr: 72%	68%	2-yr: 81%	80%		5
VUMCA I (International NPC Study Group)	1996	339	Neoadjuvant BEC × 3 cycles	5-year: 30% ($p < 0.01$)	39%	5-yr: 46%	40%		6
AOCOA (Asian Oceanian Clinical Oncology Association)	1998	334	Neoadjuvant PE × 2-3 cycles	3-yr: 42%	48%	3-yr: 71%	78%	Local control in subgroup	7
Sun Yat-Sen University, China	2001	456	Neoadjuvant PFB × 2-3 cycles	5-yr: 49% ($p = 0.05$)	59%	5-yr: 56%	63%	Local control	8
AOCOA + Sun Yat-Sen (Pooled update)	2004	784	(As above)	5-yr: 58% ($p < 0.05$)	64%	5-yr: 58%	62%	Local control and DM	9
Sapporo Medical University, Japan	2002	80	Neoadjuvant PF × 2	5-yr: 43%	55%	5-yr: 48%	60%	DM	10

DFS, Disease free survival; OS, overall survival; RT, radiotherapy arm; CT/RT, combined chemotherapy and radiotherapy arm; VCA, vincristine + cyclophosphamide + doxorubicin; PFL, cisplatin + 5-FU + leucovorin; PF, cisplatin + 5-FU; BEC, bleomycin + epirubicin + cisplatin; PE, cisplatin + epirubicin; PFB, cisplatin + 5-FU + bleomycin; (p-value) indicate significant difference.

Table 2. Randomized clinical trials of concurrent chemoradiation (with or without adjuvant chemotherapy) vs radiotherapy alone in locally advanced nasopharyngeal carcinoma

Institution	Year of Publication	Patient Number	Timing of Chemotherapy	DFS		OS		Benefit	Reference
				RT	CRT	RT	CRT		
Intergroup 0099	1998, 2001	147	Concurrent cisplatin every 3 week × 3 cycles then adjuvant cisplatin + 5-FU × 3 cyclesVCA × 6 cycles	5-yr: 29% ($p < 0.001$)	58%	5-yr: 37% ($p < 0.001$)	67%	Local control and DM	16,19
PWH-QEH (Prince of Wales Hospital and Queen Elizabeth Hospital, Hong Kong)	2002, 2004	350	Concurrent cisplatin weekly × 8 cycles	5-yr: 52%	60%	5-yr: 59% ($p < 0.05$)	70%		20,21
Taichung Veterans General Hospital, Taiwan	2003	284	Concurrent cisplatin + 5-FU × 2 cycles	5-yr: 53% ($p < 0.01$)	72%	5-yr: 54% ($p < 0.01$)	72%	Local control	22
QMH (Queen Mary Hospital, Hong Kong)	2004	219	Concurrent uracil and adjuvant cisplatin + 5-FU alternating with vincristine + bleomycin + methotrexate × 6 cycles	3-yr: 58%	69%	3-yr: 77% ($p = 0.06$)	87%	DM	11
National Cancer Center, Singapore	2004, 2008	221	Concurrent cisplatin every 3 week × 3 cycles then adjuvant cisplatin + 5-FU × 3 cycles	5-yr: 46% ($p = 0.0318$)	59%	5-yr: 49% ($p = 0.0077$)	67%	DM	12,137
Hong Kong NPC Study Group 9901	2004	348	Concurrent cisplatin every 3 week × 3 cycles Then adjuvant cisplatin + 5-FU × 3 cycles	5-yr: 55% ($p = 0.014$)	67%	5-yr: 64%	68	Local control	13,138
Sun Yat-Sen University, China	2005	115	Concurrent oxaliplatin weekly × 6 cycles	2-yr: 83% ($p < 0.05$)	96%	2-yr: 77% ($p = 0.01$)	100%	DM	23
Sun Yat-sen University, China	2008	316	Concurrent P weekly *then* adjuvant PF × 3	2-yr: 73% ($p = 0.001$)	85%	2-yr: 80% ($p = 0.003$)	90%	Local control and DM	24
Sun Yat-sen University, China	2011	230	Concurrent cisplatin weekly × 8	5-yr 95% ($p = 0.007$)	86%	5-yr 88% ($p = 0.017$)	78%	DM	25

RT, radiotherapy alone arm; CRT, concurrent chemoradiation arm; Yr, year; DM, distant metastases; (p-value) indicate significant difference.

NEOADJUVANT CHEMOTHERAPY

The advantages of neoadjuvant (induction) chemotherapy include: (1) a lower tumor load of distant micro-metastatic deposits and thus a higher chance of eradication; (2) a higher tolerance and compliance to chemotherapy in untreated patients; (3) in vivo testing of chemotherapy sensitivity by evaluating clinical response of measurable disease. The disadvantage include: (1) delay in giving definitive local treatment, favouring the growth of resistant cells and selection of partial resistance to radiotherapy; (2) accelerated tumor repopulation. These may theoretically reduce the efficacy of subsequent radiotherapy.

However, neoadjuvant chemotherapy may give a "local cooperation" within primary tumor bed by killing a few logs of cells before radiotherapy. This effect will be greater at the tumor periphery where cells are better vascularized and accessible to drug killing. Primary tumor volume reduction after neoadjuvant chemotherapy may be crucial for optimal radiotherapy delivery.

Although no significant improvement in overall survival was seen in all the published neoadjuvant chemotherapy trials,[5-10] the clinical data confirmed the theoretical expectation of neoadjuvant chemotherapy in the endpoints of progression free survival. Benefit has been seen in both local control and DM (Table 1). The probability of tumor progression before radiotherapy is shown to be remote. However, the selection and dosage of drugs is crucial, as an over-toxic schedule has been shown to impair the delivery of subsequent radiotherapy. As the VUMCA I experience strongly suggest, any possible benefit on survival may be offset by increased treatment related mortality.[6]

ADJUVANT CHEMOTHERAPY

Adjuvant chemotherapy does not delay or interfere with local treatment, but is often poorly tolerated after intensive local treatment. Seven randomized trials have tested the role of adjuvant chemotherapy.[5,11-16] However, only two studies addressed solely the use of adjuvant chemotherapy alone.[14,15] In summary, no evident benefit appears to be derived from adjuvant chemotherapy when this approach is analyzed separately. Moreover, adjuvant chemotherapy is less well tolerated, especially when concurrent chemotherapy is also given. In all the clinical trials, the compliance to adjuvant chemotherapy remains a major problem.[17] In the neoadjuvant setting, 87-100% of patients received the planned cycles of chemotherapy, while 44-93% of patients scheduled for concurrent chemotherapy received their planned cycles, and only 14-55% of patients completed their planned adjuvant chemotherapy. Chemotherapy dose intensity is most optimally maintained in the neoadjuvant setting. This disparity in dose intensities may partially explain the lack of treatment benefit associated with the administration of adjuvant chemotherapy alone.

CONCURRENT CHEMORADIATION

There are two possible mechanisms by which chemotherapy delivered concurrently with radiotherapy might affects DM. The first is a direct effect on distant micro-metastases. The second is through increased locoregional control. Clinical evidence suggested that

improved local control contributes to survival including prevention of DM arising from uncontrolled locoregional disease.[18]

A major breakthrough in the management of locally advanced NPC came about in 1998 with the publication of the pivotal phase III randomized Intergroup 0099 study.[16] This study used both concurrent and adjuvant chemotherapy combined with radiotherapy. At 3 years, the disease free survival (DFS) was 69% in the chemotherapy arm and 24% in the radiotherapy alone arm. The 3-year overall survival (OS) was 78% vs 47%, favoring chemotherapy. Updated analysis at five years confirms the benefit of chemotherapy[19] (shown in Table 2). This dramatic improvement of both DFS and OS has led to the adoption of combined modality treatment as standard of care for advanced stage NPC in the United States.

However, the radiotherapy control arm of the intergroup study has been criticized for its poor results in a heterogeneous histology mix of World Health Organization (WHO) Type I, II and III NPC patients, raising questions regarding the applicability of the results for NPC patients with mostly WHO Type II and III histology in endemic areas. Subsequently, several confirmatory studies from Asia (Hong Kong, Taiwan and Singapore) confirmed and/or supported the survival benefit of concurrent chemoradiation (with or without adjuvant chemotherapy) in advanced NPC in endemic areas.[11-13,20-25] Interestingly, three of the studies (Hong Kong,[21] Taiwan,[22] and China[23,25]) employed a purely concurrent chemotherapy regimen without the intergroup adjuvant component, and all confirmed a positive survival benefit from the use of concurrent chemotherapy alone.

A metaanalysis of individual patient data of eight randomized trials and 1753 patients further confirmed that the addition of chemotherapy to radiotherapy provides significant benefit in overall survival and disease free survival. The pooled hazard ratio of death was 0.82 (95% confidence interval (CI) 0.71 to 0.95; P = 0.006) corresponding to an absolute survival benefit of 6% at five years from chemotherapy (from 56% to 62%). A significant interaction was observed between the timing of chemotherapy and overall survival, with the highest benefit observed when chemotherapy was administered concurrently with radiotherapy.[26]

NEOADJUVANT CHEMOTHERAPY FOLLOWED BY CONCURRENT CHEMORADIATION

Since the use of both neoadjuvant chemotherapy and concurrent chemoradiation has been shown consistently to improve progression free and/or overall survival in advanced NPC, the development of sequential neoadjuvant chemotherapy followed by concurrent chemo-radiation ("neoadjuvant-concurrent" chemotherapy) would seem a logical strategy in an attempt to maximize the benefit from both approaches. In fact, this "neoadjuvant-concurrent" strategy has been pursued by several groups in Phase 2 studies and reported excellent outcome.[27-31] Our group has completed a randomized Phase 2 study of neoadjuvant docetaxel-cisplatin chemotherapy followed by concurrent cisplatin-radiotherapy (CRT) versus CRT alone in advanced NPC. We demonstrated that neoadjuvant docetaxel and cisplatin followed by CRT was well tolerated with manageable toxicity profile and allowed subsequent delivery of full dose CRT.[32] Preliminary result suggested improved survival.[32] A Phase 3 study to definitively test this strategy is warranted.

IS THERE A STANDARD CHEMORADIOTHERAPY REGIMEN (FOR EVERYONE)?

With all the available evidence, one can firmly conclude that concurrent chemoradiation (with or without adjuvant chemotherapy) is the standard of care in advanced NPC. However, due to the heterogeneity of chemotherapy protocols used in clinical trials, one cannot conclude about the superiority of one chemotherapy regimen to be combined with radiotherapy. The addition of further chemotherapy to concurrent chemoradiation, delivered in a neoadjuvant or adjuvant sequence, may further augment disease control. As evident from the metaanalysis, the treatment effect could be dependent on the timing of chemotherapy. No evidence of overall survival benefit was observed with neoadjuvant and adjuvant chemotherapy. A benefit for event free survival was, however, demonstrated in the subset of trials using neoadjuvant chemotherapy. In this group, there was an excess of treatment-related deaths in the chemotherapy group. This may suggest that if toxicity was better managed, which is the case in the more recent trials, neoadjuvant chemotherapy may play a role.[26]

Although adding adjuvant plus concurrent chemotherapy to radiotherapy conferred superior survival over radiotherapy alone in the Intergroup 0099 study, the relative contribution of concurrent and adjuvant chemotherapy has been inadequately assessed. Patients who were enrolled based on stage alone could have limited events making the studies on adjuvant chemotherapy frequently under-powered to show any benefit. The use of neoadjuvant chemotherapy is limited by the number of cycles generally permissible as definitive radiotherapy will be significantly delayed by more than six to nine weeks. These findings suggest that in the individual patient, the traditional risk profiles in therapeutic decision-making may not fully exploit all the potential therapeutic effects derived from the maximal integration of both modalities.

A RISK STRATIFICATION MODEL

One possible approach to fine tune the choice of therapy is to develop a risk stratification model, which may include other biological and molecular markers that may help to individualize the best therapeutic option.

In NPC patients, pretherapy EBV DNA in serum or plasma has been proven to correlate with cancer stage,[33] clinical outcome[34] and prognosis.[35] Posttherapy EBV DNA has even better correlation with prognosis and has been used to monitor recurrence during posttherapy surveillance.[36-38] Raised EBV DNA has been shown to predate clinical recurrence by 3 to 7 months.[30,39,40] Detectable/high level of posttherapy EBV DNA in plasma can predict a poor progression-free or overall survival when compared with those with undetectable/low DNA level,[36,37] and may be a marker of subclinical residual disease.

Targeting high-risk patients (patients with a significant likelihood of harboring occult distant metastasis, defined by residual detectable posttherapy plasma EBV DNA) using intensive chemotherapy given in the adjuvant setting may be able to reduce distant metastasis and improve survival to level of statistical significance by eradicating low-burden micro-metastasis. Sparing low-risk patients defined by the same criteria from potential chemotherapy toxicity is also an advantage. To this end, our center has initiated the use of plasma EBV DNA as a screening tool to select for NPC patients at high risk of DM at

completion of RT for enrolment into a randomized adjuvant chemotherapy trial (Hong Kong NPC study group 0502 trial, ClinicalTrials.gov number, NCT00370890).

Predictive factors (e.g., plasma EBV DNA) may be useful to stratify patients that will benefit from more intensive therapy and sparing lower risk patients from unnecessary toxicity. We have recently demonstrated that even patients with early stage NPC could be segregated by pretherapy EBV DNA levels into a poor-risk subgroup with survival similar to that of Stage III disease.[41] These patients should be candidates for more intensive therapy, as supported by clinical observation from other group.[42]

CHEMOTHERAPY IN RECURRENT OR METASTATIC DISEASE

The traditional chemotherapy drugs with activity in head and neck cancers include cisplatin, carboplatin, 5-FU, methotrexate, and bleomycin. The response rate of single agents ranged from 15% to 31%.[43] Carboplatin as single agent in NPC showed a response rate of 44% and was well tolerated.[44] Mitoxantrone demonstrated a response rate of 25% in a large multicenter Phase II trial in NPC.[45]

In head and neck cancer, combination chemotherapy regimens have consistently demonstrated higher response rate than single agent chemotherapy, and therefore recent trials have focused on the use of multi-drug combinations. Early experiences of combination chemotherapy in recurrent or metastatic NPC suggested that NPC was highly chemo-responsive and platinum containing regimen appeared to be most effective in producing complete remission.[46,47] Platinum-based combinations have consistently produced higher response rates compared with monotherapy or nonplatinum therapy[48-51] (e.g., the combination of ifosfamide, 5-FU and leucovorin[52]). Newer chemotherapy drugs included taxanes (paclitaxel and docetaxel), gemcitabine, capecitabine, irinotecan, vinorelbine and oxaliplatin, which demonstrated comparable response and generally improved side effect profile (Table 3).[23,53-73] Among the platinum-based doublets, cisplatin-gemcitabine has consistently produced both the highest overall response rate (64-93%) and complete response rate (14-21%),[59-61] which is a prerequisite for a potential cure.

More intense chemotherapy regimen combining three or more agents were attempted to improve treatment response, however often at the cost of increased toxicity and even treatment related death[74-80] (Table 4).

The natural history and management of metastatic NPC has long been an area of controversy. Distant metastases in patients with NPC have been conventionally regarded as incurable and the aim of treatment has largely been palliative. However, the experience from our center and from other investigators in the French[81] and Canadian[47] series all suggested that a small proportion of patients with metastatic NPC treated with aggressive chemotherapy had achieved long term disease free survival, suggesting the curative potential of chemotherapy, at least in a small subset of metastatic NPC.[43] For patients who developed distant metastases, we have demonstrated a marked heterogeneity in the time course and survival in different metastatic sites. In particular, patients with lung metastasis alone appeared to belong to a distinctive good prognostic group with both a longer progression free survival and overall survival.[3] We recommend an aggressive approach to manage metastatic NPC patients with good performance status, especially if the metastasis is confined to the intrathroacic site, where which long-term survival is a realistic goal after multimodality treatment.

Table 3. Platinum-based doublets or new agents in metastatic nasopharyngeal carcinoma

Investigators	Year of Publication	Patient Number	Regimen	Response Rate (%)	Complete Response (%)	Toxic Death (%)	Reference
Wang and Tan	1991	25	Cisplatin + 5-FU	76	8	4	48
Au and Ang	1994	24	Cisplatin + 5-FU	66	13	No	49
Stein et al	1996	18	Cisplatin + Ifosfamide	59	15	6	50
Yeo et al	1996	42	Carboplatin + 5-FU	38	17	No	51
Au et al	1998	24	Paclitaxel	22	0	No	53
Yeo et al	1998	27	Carboplatin + paclitaxel	59	11	No	54
Tan et al	1999	32	Carboplatin + paclitaxel	75	3	3	55
Airoldi et al	2002	12	Carboplatin + paclitaxel	25	0	0	68
Ciuleanu et al	2004	40	Carboplatin + paclitaxel	27.5	7.5	-	69
Ngeow et al	2010	30	Docetaxel	37	0	3	71
McCarthy et al	2002	9	Cisplatin + docetaxel	22	0	No	56
Chua et al	2005	19	Cisplatin + docetaxel	63	6	11	57
Foo et al	2002	25 (untreated)	Gemcitabine	28	4	No	58
		27 (pretreated)		48	3.7		
Zhang et al	2008	32	Gemcitabine	44	0	No	72
Ngan et al	2002	44	Cisplatin + gemcitabine	73	20	2	59
Ma et al	2002	18	Gemcitabine	34	6	No	60
		14	Cisplatin + gemcitabine	64	14		
Jiang et al	2005	15	Cisplatin + gemcitabine	93	21	-	61
Chua et al	2003	17	Capecitabine	24	6	No	62
	2008	49		37	6		
Ciuleanu et al	2008	23	Capecitabine	48	9	No	70
Li et al	2008	48	Cisplatin + capecitabine	62	6	No	73
Poon et al	2004	28	Irinotecan	14	0	4	63
Pan et al	2000	11	Vinorelbine	18	0	No	64
Wang et al	2006	39	Gemcitabine + vinorelbine	36	1	No	65
Ma et al	2009	42	Gemcitabine + oxaliplatin	56	2.4	No	67

Adapted from Ma BBY and Chan ATC.[66]

Table 4. Intensive chemotherapy regimens containing three or more agents in metastatic nasopharyngeal carcinoma

Investigators	Year of Publication	Patient Number	Regimen	Response Rate (%)	Complete Response (%)	Toxic Deaths (%)	Reference
Boussen et al	1991	49	Cisplatin + bleomycin + 5-FU	79	19	-	74
Su et al	1993	25	Cisplatin + bleomycin + 5-FU	40	3	12	75
Chi et al	1994	35	Cisplatin + 5-FU + leucovorin	80-100	13-15	No	76
Siu et al	1998	90	CAPABLE	41-86	6-22	8	77
Taamma et al	1999	26	Cisplatin + bleomycin + Epirubicin + 5-FU	78	35	12	78
Hasbini et al	1999	44	Cisplatin + Epirubicin + mitomycin + 5-FU	52	13	9	79
Leong et al	2004	32	Carboplatin + gemcitabine + paclitaxel	78	6	-	80

CAPABLE: cyclophosphamide + doxorubicin + cisplatin + methotrexate + bleomycin.
Adapted from Ma BBY and Chan ATC.[66]

MOLECULAR TARGETED THERAPY

Although there have been reports of long term survivors among those who achieved complete response to conventional chemotherapy,[81] recurrent or metastatic NPC remains largely an incurable disease. Better systemic agents are needed to improve the survival. In recent years, the field of cancer therapy has witnessed the emergence of novel targeted strategies that inhibit specific cancer pathways and key molecules in tumor growth and progression. With a potentially superior therapeutic index, molecular targeted agents may complement the use of conventional chemotherapy or radiotherapy in this disease.

EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

The EGFR is a transmembrane receptor tyrosine kinase of the ErbB family that is abnormally activated in many epithelial tumors. Cetuximab was the first monoclonal antibody directed at the extracellular domain of the EGFR, and was approved by Food and Drug Administration (FDA) in the treatment of advanced colon cancer in 2004, and head and neck cancer in 2006. The second class was the small molecule receptor tyrosine kinase inhibitors, including gefitinib and erlotinib, both were approved by FDA in the treatment of advanced lung cancer in 2004 and 2005.

We and others have previously demonstrated that EGFR was expressed in more than 85% of NPC biopsies.[82,83] Furthermore, high EGFR expression has been shown to be an independent predictor of poor clinical outcome in NPC.[82,83] In preclinical model, we showed that single agent cetuximab demonstrated significant anti-tumor effect in HK-1 and Hone-1 cell lines but minimal activity in CNE-2 and C666-1 cells. When cetuximab was combined with cisplatin or paclitaxel in HK-1 and Hone-1 cell lines, an additive enhancement of cytotoxic drug activity was demonstrated.[84] The activity of cetuximab was further investigated in a multi-center phase II study of cetuximab in combination with carboplatin in patients with recurrent or metastatic NPC who had disease progression after platinum-based chemotherapy. Of the 59 patients assessable for efficacy, there were seven partial responses (11.7%) and 29 patients (48.3%) with stable disease. The result showed that cetuximab in combination with carboplatin was effective in patients with recurrent or metastatic NPC who failed platinum therapy, with acceptable safety profile.[85]

Based on our previous work on the clinical activity of cetuximab in recurrent NPC, we evaluated the feasibility of adding cetuximab to concurrent cisplatin and intensity-modulated radiotherapy (IMRT) in locoregionally advanced NPC. In a phase 2 study of thirty patients, this was shown to be a feasible strategy against locoregionally advanced NPC. Preliminary survival data compare favorably with historic data and further follow-up is warranted.[86]

Single agent gefitinib were studied in two phase II clinical trials of recurrent or metastatic NPC. No clinical response was reported in both studies.[87,88] Another phase II trial was conducted to determine the efficacy of erlotinib, given as maintenance therapy after gemcitabine-platinum chemotherapy in patients with recurrent or metastatic NPC. Maintenance or second-line therapy with erlotinib after chemotherapy was not effective in this population.[89] It appeared that single anti-EGFR agent used alone was probably insufficient to arrest the growth in advanced disease.

An interesting study reported that celecoxib reduced angiogenesis and induced tumor transcriptional changes in NPC.[90] Celecoxib is a selective cyclooxygenase-2 (COX-2) inhibitor with antitumor and antiangiogenic activity. EGFR and COX-2 are involved in tumorigenesis, angiogenesis and metastases and are frequently over-expressed in NPC. There is synergistic action between COX-2 and EGFR inhibitors. An ongoing clinical trial from the same group is testing the hypothesis that combination of celecoxib and gefitinib can reduce angiogenesis and induce anti-tumorigenicity in patients with NPC (ClinicalTrials.gov number, NCT00212108).

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

Studies by us and others showed that over-expression of VEGF and its receptor VEGFR-1 (Flt-1) and VEGFR-2 (KDR) occurred in 60-90% of NPC, which was associated with lymph node metastases, distant metastases and poor survival.[91-94] Various studies have demonstrated the significant role of tumor angiogenesis in NPC disease progression.[82,95] In preclinical studies, anti-angiogenic treatment has already demonstrated promising activities in NPC.[96-98] Recently, co-expression of c-kit and stem cell factor has been demonstrated in HONE-1 NPC cells, and in primary and metastatic NPC biopsies, providing further support in the evaluation of treatment with receptor tyrosine kinase inhibitors in NPC.[99]

Sunitinib is a multi-target receptor tyrosine kinase (RTK) inhibitor against vascular endothelial growth factor receptors, platelet-derived growth factor receptors (PDGFR), c-kit and RET. In our preclinical study, we showed that single agent sunitinib demonstrated potent in vitro and in vivo growth inhibition in NPC. In vitro, sunitinib exhibited dose-dependent growth inhibition in all NPC cell lines tested with IC_{50} between 2-7.5 μM and maximum inhibition of over 97%. Sunitinib induced apoptosis and cell cycle arrest at G_0/G_1 phase. Sunitinib also moderately enhanced the growth inhibition of cisplatin or docetaxel. In vivo, single agent sunitinib demonstrated significant growth inhibition, reduced microvessel density and caused extensive tumor necrosis in a NPC xenograft model. However, concurrent administration of sunitinib and docetaxel induced severe toxicity in mice without enhanced antitumor effect. When combined with chemotherapy, sequential instead of concurrent administration schedule should be further explored.[100] We further evaluated the safety and efficacy of single-agent sunitinib in a phase II clinical trial in recurrent or metastatic NPC. Sunitinib demonstrated modest clinical activity in this heavily pretreated cohort of NPC patients. However, the high incidence of hemorrhage from the upper aerodigestive tract in NPC patients who received prior high-dose RT to the region is of concern.[101]

Given that the predominant pattern of failure in locoregionally advanced NPC treated with concurrent chemoradiation is distant metastasis, and that NPC patients with elevated VEGF have a higher likelihood of distant metastases and decreased survival, it is logical to test the addition of bevacizumab (a monoclonal antibody directed against VEGF) to the present treatment strategy for this group of patients. This strategy has been tested in a phase II study protocol conducted by Radiation Therapy Oncology Group(RTOG 0615). The preliminary result has been presented. The combination of chemotherapy, IMRT and bevacizumab in treating NPC was feasible in a multi-institutional setting. The preliminary findings seemed to suggest that bevacizumab prolongs overall survival by slowing down the progression of disease in those not cured with chemoradiation.[102]

EPIGENETIC THERAPY

Epstein-Barr virus (EBV) is a ubiquitous herpesvirus that is associated with a variety of malignancies. CpG methylation of the EBV genome plays an important role in regulating viral latency and limiting viral gene expression in normal lymphocytes and in certain tumors including Burkitt's, Hodgkin's, AIDS, and nasal lymphomas, as well as NPC. CpG methylation is implicated in silencing expression of the immunodominant EBV nuclear antigens (EBNAs-2, 3A, 3B, 3C), the latency membrane protein 1 (LMP1), lytic cycle immediate-early antigens Zta and Rta, and lytic cycle viral kinases that are implicated in the phosphorylation of ganciclovir and other antiviral nucleoside analogues.[103]

EBV's reliance on DNA methylation, and the presence of viral genomes in all tumor cells, creates a unique opportunity to specifically kill EBV-infected tumor cells. The use of DNA methyltransferase (DNMT) inhibitors, possibly in combination with histone deacetylases (HDAC) inhibitors, may allow for demethylation and reexpression of viral genes, only in the tumor cells, which can then render them susceptible to immune-mediated killing or other antiviral drugs. The utility and well-characterized nature of the EBV system makes it an ideal model system for testing such novel therapies.[104]

In patients with EBV malignancies, we undertook a clinical trial of azacitidine aimed at upregulating expression of silenced viral antigens. Analyses of several EBV promoters at the molecular level before and after treatment in patients with NPC and AIDS lymphoma show demethylation to varying degrees in all latent and early lytic EBV promoters examined, though activation of viral gene expression was observed for only one antigen by immunohistochemistry. This is the first study demonstrating that it is feasible to achieve demethylation of tumor DNA in patients with azacitidine treatment.[105] Combining azacitidine with an histone deacetylase (HDAC) inhibitor vorinostat (Suberoylanilide Hydroxamic Acid , SAHA) is being tested in an ongoing phase 1 study (NCT00336063).

IMMUNOTHERAPY

Epstein-Barr virus (EBV) is present in virtually all poorly and undifferentiated nonkeratinizing NPC regardless of geographical origin, and the viral antigens expressed by the tumor provide potential targets for immunotherapy.[106,107] Adoptive transfer of cytotoxic T cells (CTLs) specific for EBV antigens has proved highly successful as prophylaxis and treatment for EBV associated lymphoproliferative disease (PTLD) in bone marrow and solid organ transplant recipients. These highly immunogenic lymphomas arising in immunosuppressed host express all latent EBV antigens (latency Type III), including the immunodominant EBV nuclear antigen (EBNA) 3A, 3B and 3C, and are therefore ideal targets for immunotherapy. By contrast, NPC only express a restricted set of less immunogenic viral antigens (latency Type II), namely EBNA1, and latent membrane protein (LMP) 1 and 2. EBNA1 is regularly expressed in NPC. Although its processing through the HLA class I pathway is inhibited by a glycin-alanine repeat and is an unlikely target for CD8+ effectors, it is a dominant target for CD4+ T

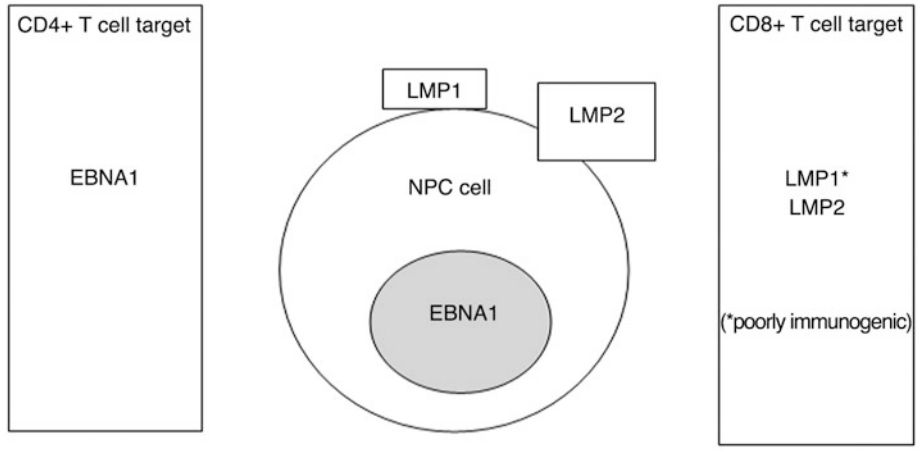

Figure 2. EBV latent protein expression in NPC cells and the immunodominant targets for CD4+ and CD8+ T-lymphocytes. (EBNA, Epstein Barr nuclear antigen; LMP, latent membrane protein).

cells. Expression of LMP1 and/or LMP2 is detectable in at least 50% of NPC tumors. LMP1 and LMP2 are both targets for CD8+ CTLs. Responses detected in healthy virus carrier indicate that LMP1 is poorly immunogenic, thus the most likely target antigen for a CD8+ CTL based therapy is LMP2 (Fig. 2).[108-110]

ADOPTIVE THERAPY

Chua et al reported the first pilot study to treat NPC using adoptive T-cell therapy in 2001.[111] Using the same approach as that employed by Rooney et al to treat PTLD, autologous EBV-transformed B-lymphoblastoid cell line (LCL) reactivated T cells were generated in vitro and used to treat four advanced cases of NPC. No adverse events occurred and infusion of CTL was associated with reduction of plasma EBV load. However, there was no evidence of tumor regression (Table 5).

The use of autologous EBV-specific CTL for NPC has since been evaluated in two clinical trials with ten patients treated in each study (Table 5).[112,113] Both studies demonstrated that autologous EBV-specific CTL is safe, induces LMP2 specific immune response and is associated with objective response and control of disease in advanced NPC. Interestingly, Comoli et al also reported the adoptive transfer of an allogeneic EBV-specific CTL in one patient with relapsed NPC resulted in temporary stabilization of disease. Local tumor biopsy showed increase in tumor infiltrating CD8 T cells.[114] In a subsequent phase 2 study, adoptive transfer of EBV-specific T cells results in sustained clinical responses in patients with locoregional nasopharyngeal carcinoma.[115] Taken altogether, the result of these studies showed that it is feasible to boost EBV-specific immune response in NPC patients and provide further rationale to explore EBV as a target for immunotherapy.

Table 5. Clinical studies of immunotherapy targeting EBV in nasopharyngeal carcinoma

Investigator (country)	Patient Number	Treatment	Results	Reference
Chua et al (Hong Kong)	4	Autologous EBV specific CTL	4 NR Increase in EBV specific CTL precursor frequency Reduction in plasma EBV burden	111
Straathof et al (US)	10	Autologous EBV specific CTL	*Refractory disease*: 2 CR (remain in remission 11-23 months post infusion), 1 PR, 1 SD, 2 NR *Adjuvant treatment for advanced disease*: 4 patients remain in remission 19-27 months post infusion Decrease of EBV viral load in 6/9 2-fold increase of LMP2-specific T cells in 4/8	112
Comoli et al (Italy)	10	Autologous EBV specific CTL	2 PR, 4 SD, 4 PD Increased frequency of EBV specific immunity in all Appearance of LMP2-specific responses in 4 patients (3 of whom had clinical benefit)	113
Comoli et al (Italy)	1	Allogeneic EBV specific CTL	SD Increase in tumor infiltrating CD8+ T-lymphocytes Long term increase of LMP2-specific imunity	114
Lin et al (China)	16	LMP2 peptide pulsed autologous dendritic cell vaccination	2 PR, 14 PD Peptide specific CD8+ T-cell response elicited or boosted in 9/16	116

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NR, no response.

VACCINATION

A vaccine consisting of dendritic cells pulsed with peptides derived from LMP2 has been evaluated in 16 NPC patients with local recurrence or distant metastasis after conventional treatment.[116] Peptide specific T-cell response were elicited or boosted in nine patients and partial tumor reduction was observed in two patients (Table 5). Currently, a vaccination trial is ongoing in UK and Hong Kong using a modified vaccinia virus expressing an EBNA1-LMP2 fusion protein (MVA-EL) to elicit CD4+ and CD8+ T cell against the two EBV proteins expressed in NPC patients.[117] The phase 1 trial showed that MVA-EL is safe and immunogenic at all doses tested. The highest dose is the most immunogenic and is recommended for phase II trials testing clinical benefit.[118] Alternatively, an LMP-based polyepitopte vaccine has also been developed for EBV associated Hodgkin disease and NPC.[119,120]

FUTURE PROSPECT FOR EBV TARGETED IMMUNOTHERAPY IN NPC

Several factors suggest that EBV targeted immunotherapy may be successful in treating NPC: (1) the tumor expresses EBV proteins that are known targets for CD8+ and/or CD4+ T cells. (2) T-cell response to these viral proteins is restricted through HLA alleles present at high frequency in the patient population. (3) Antigen processing pathways within the malignant cells appear to be intact. (4) Strategies have been developed to selectively reactivate the appropriate CTL response.[106]

In the reported clinical trials, EBV-specific CTL lines were generated by stimulation with EBV-LCL, which favoured the outgrowth of CTL responses to the immunodominant EBNA3 proteins rather than the subdominant EBV proteins LMP1 and LMP2 expressed in NPC. Antitumor response could be further enhanced by strategies to increase the specificities of CTL lines for the EBV latency II antigens expressed in NPC,[117,119-121] and to improve the in vivo expansion of adoptively transferred CTL.[122,123] Table 6 summarized the current strategies to improve EBV targeting immunotherapy for NPC.[105,107,112,113,116,117,119-122,124-128]

Table 6. Improving cellular immunotherapy targeting EBV in nasopharyngeal carcinoma

Strategies	Reference
1) Enrichment of subdominant EBV antigen specificities:	
a) stimulation with dendritic cells expressing LMP2, LMP1 and/or EBNA1	107,121
b) expansion of LCL-stimulated CTL after vaccination of patients with MVA-EL vaccine, LMP1 polyepitope adenovirus vaccine, or LMP2 peptide pulsed dendritic cells.	116,117,119,120
2) Inducing expression of immunodominant EBV antigens in tumor cells	
a) demethylating agent	105
b) chemotherapy	124
c) radiation	125
3) Higher CTL doses	113,122
4) Co-administration of IL-2	113
5) Host preconditioning by lymphodepletion chemotherapy	122,126,127
6) Utilize earlier in the disease course of NPC	112

GENE THERAPY

The ideal therapeutic target for gene-based therapy would be one which is commonly abnormal in tumor but also bear prognostic value. The challenge in NPC is that only a few genetic abnormities have been identified, which might also bear some prognostic significance.[129] The early studies in NPC exploited the therapeutic potential of p53 and p16 gene, and achieved limited success.[130-133] More recently the human endostatin gene therapy has been investigated in NPC.[97,98] However, the major challenge is to achieve tumor-specific expression and cytotoxicity.

A unique feature of NPC is its almost universal association with the EBV, which is expressed in a latent form exclusively in cancer cells, and not in the surrounding normal tissues. One approach to targeted expression of therapeutic genes by exploiting the presence of EBNA1 in NPC was pursued by the group led by Fei-Fei Liu. They have constructed a novel replication deficient adenovirus vector (ad5.oriP) in which transgene expression is under the transcriptional regulation of the family of repeats domain of the origin of replication (oriP) of EBV. Utilizing p53 as the therapeutic gene (ad5.oriP.p53), selective cytotoxicity was achieved only in EBV positive NPC cells, which was enhanced with the addition of radiation.[134] To achieve both tumor specificity and improved vector distribution, the same group has also established a conditionally replicating adenovirus (adv.oriP.E1A) for NPC gene therapy.[135] This conditionally replicative adenovirus strategy can be combined with additional therapeutic genes, such as the FasL,[136] for possible systemic delivery. However, an important safety issue that remains to be addressed is the reservoir of EBV in memory B-lymphocytes. Despite these proof-of-principle studies demonstrated in the laboratory, cancer gene therapy is still in infancy at the moment and its clinical efficacy remains to be tested in NPC patients.

CONCLUSION

NPC has traditionally been treated by local radiotherapy with great success especially for early stage disease. The recognition of a high rate of distant metastases leads investigators to explore the incorporation of systemic therapy (principally chemotherapy) into the primary radiotherapy. Concurrent chemoradiation with or without adjuvant chemotherapy has been shown to improve the overall survival and disease free survival when added to radiotherapy in advanced NPC, and becomes the standard of care. The sequence of neoadjuvant chemotherapy followed by concurrent chemoradiation allows better maintenance of chemotherapy dose intensity, and has demonstrated excellent result in several phase II studies, and would be a reasonable approach to be explored in future randomized trials. The availability of plasma EBV DNA as sensitive molecular markers of residual disease after radiotherapy opened up new opportunity for adjuvant therapy to target only the high risk group. The incorporation of newer, less toxic and more effective anticancer agents such as the taxanes, gemcitabine or molecular targeted agents into combined modality regimens warrant continued exploration. With the increased understanding of the molecular and immune mechanism in NPC and its unique association with EBV, new therapy options with much reduced toxicity can be developed. Exploration of targeted agents against EGFR and VEGF appeared most promising. EBV directed immunotherapy with adoptive transfer of refined CTL and EBV vaccine specifically targeting EBNA1/LMP1/LMP2 may be the road to the future in this fascinating EBV-associated epithelial cancer.

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CHAPTER 11

NASOPHARYNGEAL CARCINOMA IMMUNOTHERAPY: Current Strategies and Perspectives

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Abstract: Recent success in treating Epstein-Barr virus (EBV)-associated posttransplant lymphoproliferative disorder (PTLD) using cytotoxic T-cell (CTL) based immunotherapy has led to interest in the development of CTL-based immunotherapy to treat other EBV-associated malignancies, including Nasopharyngeal carcinoma (NPC). However unlike PTLD, which arises in immunosuppressed individuals following transplant, NPC can arise in immunocompetent individuals, expresses a limited array of EBV antigens that are poorly immunogenic, and appear to suppress the function of these T cells either directly or through the expansion of regulatory T cells. There is therefore a unique set of problems that need to be addressed in order to optimise CTL-therapy for the effective treatment of NPC.

INTRODUCTION

The primary function of CTL is to recognise and clear both intracellular pathogens and malignant cells.[1,2] Following T-cell receptor engagement of a peptide-major histocompatibility (MHC) class I complex on the surface of an infected or malignant cells, CTL function by inducing lysis of the target cell via a number of molecular pathways.[3] The potential development of CTL-based immunotherapy offers an attractive, low-toxicity alternative to the use of current therapies employed to treat a number of human malignancies, including NPC. The consistent detection of EBV in NPC offers a potential target for CTL-based therapeutic treatment of NPC.

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Following the control of primary lytic infection in B cells, EBV causes a persistent life-long latent infection, characterised by the expression of the EBV nuclear antigens (EBNA1-3) and the latent membrane proteins (LMP 1 and 2). It is now evident that latent infection is controlled by a population of CTL that recognise epitopes derived from these antigens.[4] Furthermore, dysfunction in this CTL population, either through immunosuppression, which can lead to PTLD in transplant patients following immunosuppressive therapy, or via loss of function, which appears evident in EBV-associated Hodgkin's lymphoma (HL) and NPC, can result in the uncontrolled growth of EBV-transformed malignant cells.[5,6] Conversely, augmentation of the CTL response against the latent antigens offers a potential therapy to treat EBV-associated malignancies. CTL-based therapy has thus far been successfully employed to treat PTLD[7] and a number of strategies are currently being investigated as an alternative treatment for NPC, particularly for the treatment of patients who are unresponsive to current therapies.

IMMUNOLOGICAL TARGETS IN NPC

NPC cells do not express the full array of latent antigens, as typically occurs in PTLD. Together with EBV-associated HL, NPC represents a Type II latency malignancy, whereby antigen expression is limited to LMP 1 and 2 and EBNA1.[7] Therefore immunotherapeutic approaches employed to treat EBV-associated NPC are dependent upon the capacity to generate an immunological response against these antigens, which play a significant role in EBV latency and have evolved to evade immune recognition.

LMP 1 and 2 play a role in activating and transforming cells following infection, allowing proliferation and survival of latently infected cells.[8,9] The LMP antigens are thus oncogenic by nature. Furthermore, the LMP antigens, particularly LMP1, are poorly immunogenic, likely due to poor antigen processing in infected cells and the subsequent limited amount of antigen available for presentation by MHC class I molecules.[10] As a consequence, the LMP antigens, particularly LMP1, generate a subdominant CTL response when compared to the responses generated against lytic cycle antigens and other latent antigens, such as EBNA3.[2] Evasion of the immune response and the subsequent minimalisation of the LMP-specific CTL response may play some role in the capacity of LMP1 and 2 bearing malignancies to occur. Accordingly, amplification of the LMP-specific CTL response offers an obvious choice when developing an immunotherapeutic treatment for NPC.

In contrast to the LMP antigens, which are not detectable in all EBV-associated malignancies, EBNA1 can be detected in all EBV-associated malignancies.[7] EBNA1 has been shown to be highly stable and contains a glycine-alanine repeat sequence near its N-terminus that inhibit translation and subsequent self-replication.[11-13] Consequently, EBNA1 is processed poorly via the MHC class I pathway. However, since the discovery of EBNA1-specific $CD8^+$ CTL, which were thought to be induced via cross-presentation by professional antigen presentation cells rather than via direct recognition of infected cells,[14] it has been clearly established that endogenously processed EBNA1 can be detected by $CD8^+$ T cells.[15-17] This has lead to the realisation that in addition to the LMP proteins, EBNA1 may be a viable target for CTL-based immunotherapy of NPC.

CTL-BASED IMMUNOTHERAPEUTIC TARGETING OF LMP 1 AND 2 AND EBNA 1 IN NPC TUMORS

It is well established that CTL-based therapies can be employed successfully to treat PTLD in immunosuppressed transplant patients.[7] The expansion of EBV-specific $CD8^+$ CTL to treat PTLD employs EBV-transformed lymphoblastoid cells lines (LCLs) as the antigenic source. A number of studies have used a similar strategy to treat NPC patients, see Table 1.[18-23] These studies have established the safety profile of using CTL therapy for the treatment of NPC and have shown some clinical efficacy, particularly for the treatment of locoregional disease.[21] However, the predominance of T cells specific for the EBNA3 antigens and the failure to generate LMP1 and 2 and EBNA1 specific T cells from some donors using LCL-mediated expansion is likely to limit the efficacy of this treatment for NPC. Strategies aimed at optimising the generation of CTL specific for LMP1 and 2 and EBNA1 should therefore be beneficial when developing a CTL-based treatment for NPC. Current strategies used to improve specificity in CTL lines include individual peptides, polyepitope technology and whole or truncated antigens; see Figure 1 for expansion techniques used to generate CTL.

Peptide-based expansion utilizes the simplest strategy to expand CTL specific for target antigens using known peptide epitopes, either singularly or pooled.[24,25] Due to the oncogenicity and poor immunogenicity of LMP1 and LMP2A, the use of minimal $CD8^+$ T-cell epitopes is ideally suited for the generation of specific CTL. LMP2A, and to a lesser extent LMP1, encoded epitopes have been identified across a broad range of HLA types, making defined epitopes practical for use in the treatment of NPC across a range of HLA-types.[26]

Although peptide-based expansion offers an attractive method to produce CTL for adoptive immunotherapy into NPC patients, the capacity to produce a cost-effective therapy based on defined LMP1 and 2, and possibly EBNA1 CTL epitopes, may be limited by the expense of producing individual peptides tailored to different HLA types. In addition treatment with a poly-specific population of CTL offers a more attractive proposition by broadening the number of epitopes and antigens targeted, thus limited any effects mutations in an individual epitope sequence could have upon efficacy. One polyvalent approach currently under development to treat NPC involves the use of a LMP-specific CTL polyepitope, which is produced by linking peptides encoding CTL epitopes, targeting a broad-range of HLA types, consecutively in a single polypeptide.[27,28] The current method employed to deliver the LMP polyepitope consists of a modified adenoviral delivery vector, encoding the LMP1 and 2 polyepitope linked to the EBNA1 gene, without the glycine alanine repeat (EBNA1ΔGA), known as AdE1-LMPpoly. Comparison with LCL-mediated expansion confirmed that stimulation with AdE1-LMPpoly optimised the generation of CTL capable of recognising LMP1 and 2 and EBNA1 (Fig. 2), thus targeting antigens expressed in malignant cells and offering a more cost-effective alternative to the use of single peptide-based expansion strategies to generate CTL for the treatment of NPC.[28] We have recently completed a phase I clinical study in Hong Kong investigating the safety of adoptive transfer of AdE1-LMPpoly generated T cells in recurrent NPC patients.[63] The adoptive transfer of AdE1-LMPpoly T cells was safe, well-tolerated and induced disease stabilisation in the majority of patients (Table 1).

Another approach currently under investigation to generate CTL to treat NPC employs the use of delivery vectors encoding whole or partial antigens.[29] This offers

Table 1. Current or completed trials using CTL-based immunotherapy to treat NPC

Location of Trial	Patients	Strategy for CTL Generation	LMP/ EBNA1-Specificity in Infused CTL	Concurrent Treatment	CTL Dose	Results/Comments/ Reference
Hong Kong	4—disease	LCL stimulation	Not tested	None	1 dose of 5×10^7 - 3×10^8	No discernable anti-tumour efficacy[20]
Houston, USA	4—remission 6—relapsed or refractory disease	LCL stimulation	3 cell lines from patients in remission and 5 patients with disease had detectable LMP2A-specific CTL	None	1-2 doses. 2×10^7 - $2 \times 10^8/m^2$	All remission patients remained in remission at least 19mo posttherapy 2 patients in complete remission at least 11mo posttherapy PR in 2 patients at least 4 mo posttherapy, then relapsed[19]
Milan, Italy	10—disease	LCL stimulation	5 cell lines had detectable LMP2A-specific CTL	Low dose recombinant IL-2	2-23 doses. 2×10^7 - 8×10^7 CTL/m^2	PR in 2 patients at least 3 months post-therapy[18]
Houston, USA	8—disease	LCL stimulation	5 cell lines had detectable LMP2A-specific CTL	Anti-CD45 mAb prior to CTL	2×10^7 - 1×10^8 CTL/m^2	SD in 2 patients at least 12 months post therapy CR in 1 patient 24 months posttherapy[22]

continued on next page

Table 1. Continued

Location of Trial	Patients	Strategy for CTL Generation	LMP/ EBNA1-Specificity in Infused CTL	Concurrent Treatment	CTL Dose	Results/Comments/ Reference
Houston, USA	8—remission 3—locoregional disease 11—metastatic disease	LCL stimulation	17 cell lines—LMP2A specific T cells 5 cells lines—EBNA1-specific T cells 7 cell lines—low -LMP1-specific T cells	None	2 doses. 2×10^7 – 2×10^8 CTL/m^2	5 of 8 patients remained in remission 25 to 82 months post-infusion CR in 2 of 3 patients with locoregional disease, PR in the other patient CR in 2 of 11 patients with metastatic disease, PR in 1 patient, SD in 2 patients[21]
Milan, Italy	11—disease	LCL stimulation	6 cell lines had detectable LMP2 responses	Cyclophosphamide and fludarabine prior to therapy; Low dose IL-2	2 doses 1.6 - 5×10^8	SD in 3 patients 4-16+months post therapy PR in 2 patients 5-8 months post therapy[23]
Boston, USA	10—disease	LCL stimulation	CTL generated from 9 patients—7 patients received CTL	None	2 doses: 2×10^7 and 1×10^8, or $2 \times 1 \times 10^8$.	SD in 1 patient up to 64 weeks post therapy[62]
Brisbane, Australia, and Hong Kong	25—recurrent disease 25—remission	Single stimulation with AdE1-LMPpoly	LMP/EBNA specific CTL generated from 16 of 22 recurrent patients.	None	Up to 6 doses of 2×10^7 - 4×10^7	SD in 10 of 14 treated recurrent disease patients 38-420 days post therapy[63]

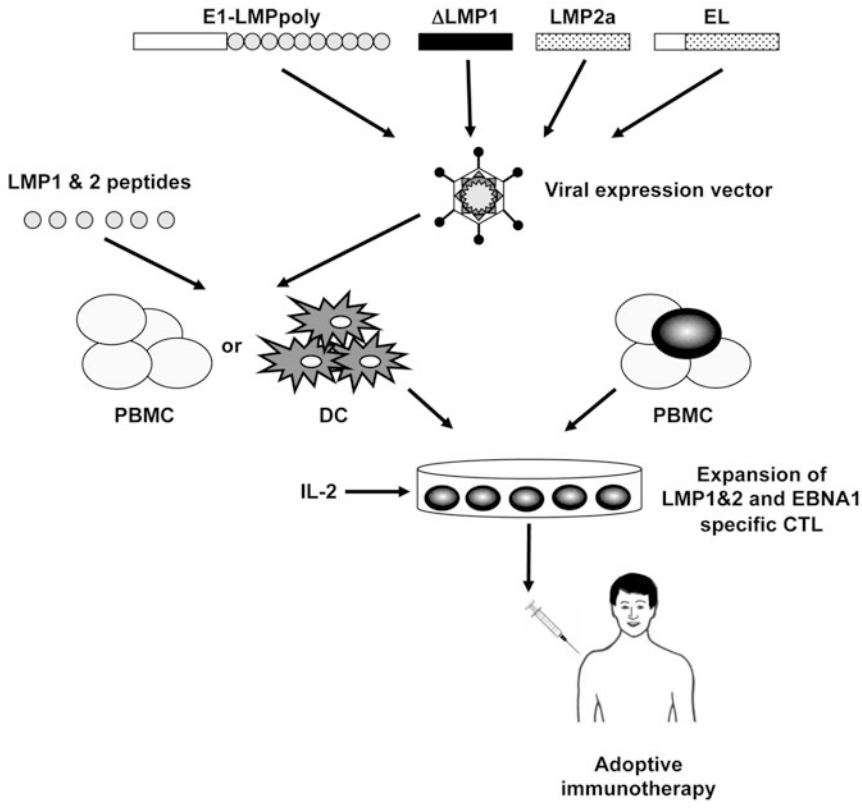

Figure 1. T-cell-based adoptive immunotherapy for NPC. PBMC from the patient are stimulated with autologous PBMC or DC pulsed with LMP peptides or infected with a replication deficient viral vector encoding a polyepitope or whole antigen. Following stimulation these T cells are assessed for antigen specificity and stored for future adoptive immunotherapy.

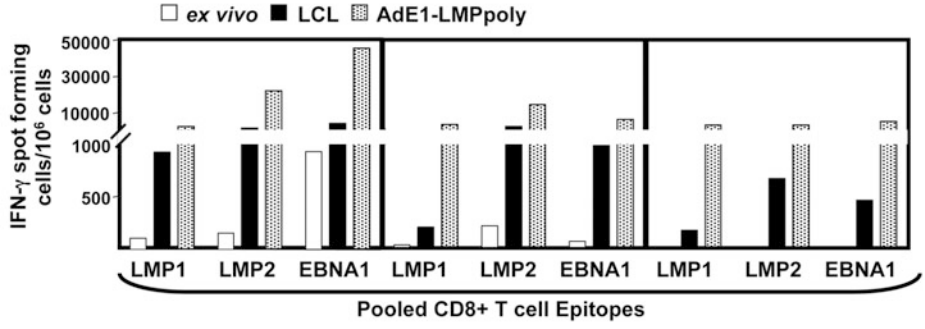

Figure 2. In vitro expansion of EBV latent membrane protein specific T cells using a recombinant polyepitope vaccine. Comparison of in vitro T-cell expansion from three donors without stimulation (ex vivo) or following stimulation with autologous LCL or E1-LMPpoly. Data represents the number of IFN-γ producing cells responding to pooled LMP1, LMP2 or EBNA1 CD8+ peptide epitopes following in vitro recall (Elispot assay).

some advantages over treatment using defined epitopes because knowledge of all of the encoded CTL epitopes is not absolutely necessary, and possibly by broadening the specificity of the responding CTL. However, the use of whole or partial antigens requires knowledge of sequences with oncogenic potential, which likely need to be removed, and may require more complex delivery vehicles or multiple delivery vehicles to deliver all of the antigens in question. In addition to the use of EBNA1ΔGA in the AdE1-LMPpoly, expansion of CTL with full-length LMP2A and a truncated LMP1, which contains a 44 amino acid deletion at its N-terminus to reduce toxicity, is currently being explored.[30,31] Similar to the E1-LMPpoly construct, both of these antigens have been expressed using a modified adenoviral vectors and have been shown to be effective in generating LMP-specific CTL in vitro from the PBMC of healthy donors. These adenoviral vectors encoding full length antigens have been used successfully in clinical trials to treat EBV-associated lymphoma's.[32] Another strategy currently being developed employs the C-terminal domain of EBNA1 conjugated to LMP2A. Expression using a modified Vaccinia Ankara (referred to as MVA-EL) has been employed successfully to present LMP and EBNA1-restricted epitopes to specific CTL.[33]

AN IMMUNOTHERAPEUTIC VACCINE TO TREAT NPC

The development of a vaccine offers another potential more cost-effective immunotherapeutic treatment for NPC, particularly in regions where the expense of CTL-based therapy may be prohibitive. Currently, the polyepitope-based vaccine is being assessed for its capacity to generate LMP-specific CTL following vaccination. Studies using vaccination of HLA A2/kb transgenic mice have shown that the polyepitope can be utilized to generate LMP-specific CTL following vaccination.[27] Clinical trials have also commenced investigating the use of LMP2A encoded peptide-based vaccines to generate CTL (Table 2). In a completed study, the vaccination of NPC patients using dendritic cells pulsed with peptides generated an expansion in the LMP-specific response in the peripheral blood in the majority of patients, and a partial clinical response in 2 of 16 patients enrolled in the study.[34] Vaccine approaches are also in development using whole antigens. A recently completed Phase II study evaluated the use of dendritic cells transduced with an adenovirus-ΔLMP1-LMP2 vector.[35] This study demonstrated the safety of this strategy, however no increase in the frequency of LMP1/2-specific T cells was detected. It is likely that the direct immunisation with recombinant viral vectors will lead to a more efficient induction of T-cell responses. The commencement of a clinical trial using the MVA-EL vaccine (Table 2), should provide evidence for the effectiveness of the direct administration of a poly-specific vaccine to generate LMP/EBNA1-specific CTL responses in patients. Preliminary observations from this study have demonstrated an increase in EBNA1 and/or LMP2 specific T-cell responses in 15 of 18 patients.[36]

THE POTENTIAL IMPACT OF IMMUNE EVASION ON CTL-BASED IMMUNOTHERAPY

It remains to be definitively shown if the LMP and EBNA1-specific CTL generated using the aforementioned approaches have the capacity to recognise and kill NPC

Table 2. Current or completed vaccine-based trials for NPC

Location of Trial	Patients	Vaccination Strategy	LMP/EBNA1 Specific Immune Response	Results/Comments/ Reference
Queen Mary Hospital, Hong Kong	16	LMP2A-specific peptide pulsed dendritic cells	T-cell response elicited in 9 patients	PR in 2 patients[34]
NIH, Bethesda, USA	99—In remission with a high risk of recurrence	LMP2A-specific peptides mixed with Montanide ISA-51	Not available	http://www.clinicaltrials.gov/ct/show/NCT00078494
National Cancer Centre and Singapore General Hospital, Singapore	16—metastatic NPC	DC-transduced with DLMP1-LMP2	No detectable LMP1/2 responses	PR in 1 patient, SD in 2 patients[35]
University of Birmingham, UK. Chinese University of Hong Kong, Hong Kong	18—12 weeks post primary therapy.	MVA-EBNA1/LMP2	Significant increase in responses to EBNA1 and/ or LMP2 in 15/18 patients	No data available on efficacy[36]

cells. Immune evasion, which likely plays a critical role in the development of NPC, may also significantly impact upon the efficacy of CTL-based therapy. Low levels of antigen expression and poor processing of these antigens may limit presentation in malignant cells,[10] providing one possible mechanism for immune evasion. Although recent reports have suggested a down-regulation in molecules associated with the MHC class I pathway in NPC cells, including the HLA molecules,[37] analysis of the antigen presentation capacity in NPC cells lines has demonstrated that these cells retain the capacity to present peptides to CTL via MHC class I.[38,39] However there is only limited evidence demonstrating presentation of LMP1, 2 or EBNA1 by NPC cells.[18] Analysis of the capacity of NPC cell lines, and possibly more importantly, tumour cells directly from biopsies, to present LMP1 and 2 and EBNA1 may be critical in defining parameters to generate successful CTL-based immunotherapy. High avidity CTL may be required to recognise low level antigen presentation in NPC cells. In addition, it has been established that all three antigens are not always detectable in NPC.[37] Therefore, knowledge of the antigen expression pattern in malignant cells may provide valuable information into the efficacy of CTL therapy in patients at different stages of disease. However, the capacity to detect antigen expression in malignant cells is likely to be inferior to the ability of CTL to recognise antigen presented via MHC class I, as such CTL therapy may still be applicable in cases where the target antigen is difficult to detect. Furthermore, the implementation of a strategy that generates CTL against all three antigens, such as with a polyepitope or whole antigens, may avoid problems associated with down-regulation in the expression of a particular antigen by generating CTL which can recognise the expressed antigens.

In addition to the limitations associated with antigen presentation in malignant cells, the impact tumour infiltrating regulatory T cells (T_{reg}) have upon CTL function may also need to be addressed. As has become evident in a number of malignancies, including other EBV-associated malignancies such as Hodgkin's lymphoma,[5] NPC is associated with an increase in peripheral T_{reg} cells and the presence of T_{reg} cells in the tumour infiltrate.[40] It is probable that infiltrating T_{reg} cells protect malignant NPC cells from immune recognition by suppressing the function of CTL and other infiltrating cells (Fig. 3). Similarly, following adoptive immunotherapy, it is possible that T_{reg} cells may impact upon the ability of transferred CTL to kill NPC cells. Murine cancer models have demonstrated that the depletion of tumour-infiltrating T_{reg} cells can significantly enhance protection.[41,42] Chemotherapeutic lymphodepletion has been proposed as one mechanism to deplete T_{reg} cells in humans.[43,44] A clinical trial employing two monoclonal antibodies directed against CD45 as a lymphodepletive agent prior to CTL transfer demonstrated the efficient reconstitution of EBV-specific CTL.[22] However, more recent observations using cyclophosphamide and fludarabine demonstrated no clinical benefit in lymphodepleting chemotherapy.[23]

Other immune evasion strategies mediated by the malignant cells themselves may also impact upon the efficacy of immunotherapeutic approaches to treat NPC. Malignant NPC cells have been shown to secrete Galectin-9, which at high concentrations induces the apoptosis of activated EBV-specific T cells following interaction with T-cell immunoglobulin mucin-3 (TIM-3).[45] In vitro expanded LMP1 and 2 and EBNA1 specific T cells, which upregulate TIM-3 following activation may be susceptible to Galectin-9 mediated apoptosis following adoptive transfer. Therefore, strategies to limit Galectin-9 and other immune evasion strategies of the malignant cells may be necessary in order to optimise the effectiveness of adoptive T-cell therapy.

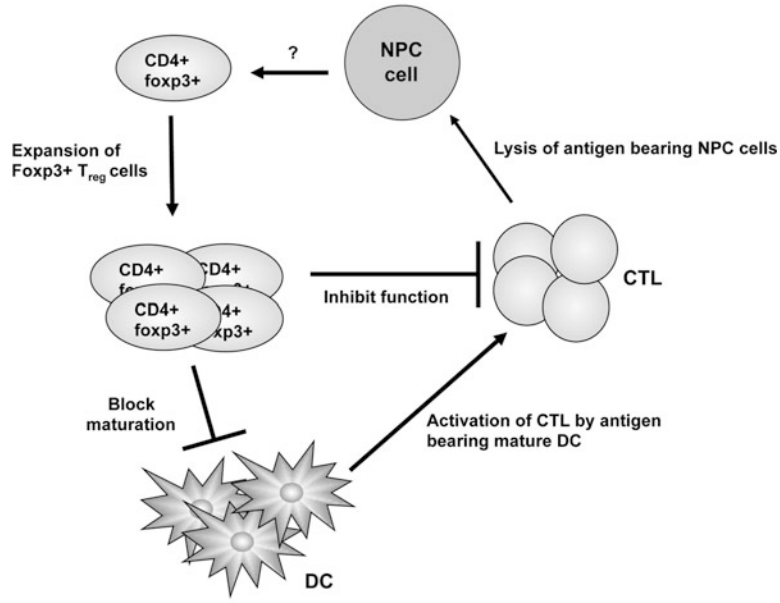

Figure 3. Role of T_{reg} cells in immune evasion by NPC cells. T_{reg} cells, possibly generated following direct contact with tumour cells or via tumour-produced cytokines/chemokines, are thought to mediate the down-regulation in function of antigen presenting cells, such as DCs, preventing CTL activation or suppress the function of CTL directly.

A ROLE FOR CD4+ T CELLS IN CTL-MEDIATED IMMUNOTHERAPY OF NPC

It is now well established that CD4+ T-cell play a critical role in enhancing the cell-mediated immune responses that are required to clear both viral infections and malignant cells.[46] They primarily function as helper T cells by producing cytokines, including interferon-γ, which up-regulates antigen presentation and cytokine production in professional antigen presenting cells and other phagocytic cells; and IL-2 which activates T cells. Both of these functions play a critical role in the activation of CTL. In addition there is evidence that CD4+ T cells can also exhibit the type of cytolytic functions, such as lysis of infected target cells, that are typically associated with CD8+ CTL.[47,48] The development of an immunotherapeutic strategy to treat NPC that induces specific CD4+ T cells may therefore provide some benefits in modulating the immunological environment to the advantage of CTL. Although not as thorough as the analysis of the CD8+ T-cell response to the NPC-associated antigens, a number of reports have detected CD4+ T-cell responses directed against LMP1 and 2 and EBNA1.[48-51] In addition, we have some preliminary evidence that CD4+ T cells can be expanded from NPC patients using the AdE1-LMPpoly vector and that vaccination of HLA A2/Kb transgenic mice with AdE1-LMPpoly can lead to the induction of a specific CD4+ T-cell response (Smith and Khanna, unpublished observations). Similarly, the MVA-EL construct has also been used successfully to present antigens to EBNA1-specific CD4+ T cells, in addition to presentation to EBNA1 and LMP2A-specific CD8+ T cells.[33]

CONCLUSION

Although current therapies are highly successful in treating NPC, survival rates are reduced in patients with advanced disease.[52,53] In addition, current therapies can produce side-effects associated with the toxicity of the radio/chemotherapeutic agent employed.[53] An alternative therapy, such as the use of CTL-based immunotherapy which specifically targets malignant cells, offers an attractive prospect for the treatment of incurable NPC, and to minimize toxic side-effects associated with current therapies. However, it remains to be elucidated if LMP and EBNA1 specific T cells will be capable of clearing malignant cells. Aside from those issues raised above, primarily associated with immune evasion, a number of other factors may significantly influence the effectiveness of CTL-mediated therapy in NPC patients, including the capacity to generate CTL from disease patients that will survive following transfer and that have the capacity to home to the sites of disease.

Although CTL generated in tissue culture display the typically effector-phenotype associated with CTL, such as production of IFN-γ and lysis of antigen-bearing targets,[18,19,28] CTL can be heterogeneous, primarily with regard to their differentiation status and homing properties. Following antigen encounter, a naïve or memory T cell will proliferate, and acquire an increasing number of effector functions, resulting in fully differentiated effector cells which display the full array of effector functions.[54,55] However differentiation into effector cells significantly alters the trafficking properties of the T cell.[56] There is now evidence that this change in homing properties can be tissue-specific, whereby stimulation in different lymphoid organs can influence trafficking to particular peripheral tissues.[57,58] The effective immunotherapeutic treatment of NPC, either with adoptive therapy or vaccination may be dependent upon the capacity to generate CTL that can home to the nasopharyngeal tissue and other sites of metastatic disease. It also remains to be elucidated what impact the differentiation status of CTL has upon survival posttransfer. Although terminal differentiation may generate greater effector function, poor survival of these T cells posttransfer may reduce the number of cells accessing tumour sites. There is evidence that less differentiated T cells retain a greater capacity to expand following antigen encounter in vivo and provide greater protection following transfer.[59] Therefore, treatment with nonterminally differentiated CTL may have some benefit in prolonging their survival and proliferation capacity following adoptive transfer. Current strategies used to generate CTL that rely upon long-term in vitro cultures will generate cells with a late-stage effector phenotype.

Lymphodepletion prior to adoptive transfer may provide another mechanism to enhance survival and proliferation of transferred CTL. In addition to the benefits associated with the removal of T_{reg} cells, there is evidence that lymphodepletion can enhance the efficacy of CTL-based therapy by removing T cells which compete for homeostatic cytokines, such as IL-15 and IL-7, and thus creating space in the lymphoid system to accommodate transferred T cells.[42,44] However, recent observations have suggested that whilst lymphodepletion may promote T-cell engraftment,[22] it may not improve the clinical outcome following T-cell therapy.[23]

One challenge that still may need to be overcome prior to the effective implementation of a NPC-specific CTL-therapy is whether or not LMP and EBNA1-specific CTL can be generated from all NPC patients. Evidence generated from completed trials suggests that current expansion strategies are not effective at inducing specific CTL from all patients. Although more specific stimulation strategies may improve the specificity

of CTL lines, other factors may impact upon the ability to expand CTL from patients. A number of these factor are likely to be associated with the diseased state of the patient, such as a possible loss of antigen specific T cells and any inhibitory effects caused by an increased population of T_{reg} cells. Additionally a low precursor frequency of LMP and EBNA1-specific T cells in the peripheral blood may limit the generation of enough CTL. An alternative therapy, exploiting the generation of a bank of cryopreserved HLA-matched specific CTL, may offer another option as a source of CTL to treat NPC patients. A cryopreserved CTL bank has been generated using LCL-mediated expansion for the treatment of EBV-associated diseases.[60,61] A similar strategy could be applicable for NPC and may offer additional advantages by avoiding the delay in treatment that would occur when generating autologous CTL.

Given the outlined recent findings, it is evident that use of a CTL-based therapy alone is unlikely to provide the optimal therapeutic option for the treatment of NPC, particularly for bulky recurrent metastatic disease. Nevertheless, preliminary evidence suggests that CTL-therapy is well tolerated and can improve the clinical outcome for some NPC patients. Ultimately, the employment of LMP and EBNA1 poly-specific CTL, possibly in combination with helper T cells, following prior chemotherapeutic treatment to reduce tumour burden, deplete T_{reg} cells and provide space in the lymphoid compartment, may offer the most attractive option for the implementation of CTL-therapy to treat NPC, particularly when current therapies alone are unable to control the spread of disease.

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CHAPTER 12

THERAPEUTIC INDUCTION OF APOPTOSIS IN NASOPHARYNGEAL CARCINOMA

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Abstract: Apoptosis is a mechanism of cell death that is pivotal for the maintenance of cellular homeostasis within the human body. Not surprisingly, mutations rendering cells resistant to apoptosis are acquired in virtually all cancers. A full understanding of such mutations is important for the development of clinically successful therapeutic strategies.

In nasopharyngeal carcinoma (NPC), inhibition of both receptor- and mitochondrial-mediated apoptosis is achieved through the inter-related expression of human and Epstein-Barr Virus (EBV) genes. In particular, the over-expression of NF-κB, mediated in part by EBV LMP1, may be the central mechanism leading to the expression of several anti-apoptotic genes, including survivin, Bfl-1, Bcl-2 and A20. This biological insight has already facilitated the development of several strategies to directly overcome apoptosis resistance, many of which aim to directly modify Bcl-2 family protein expression.

In this chapter, we will summarize the heretofore elucidated mechanisms of resistance to apoptosis in NPC. We will also examine therapeutic strategies directly targeting apoptosis in NPC that have been developed thus far.

INTRODUCTION

Apoptosis is a mode of programmed cell death characterized by pyknosis (chromatin condensation), nuclear fragmentation, membrane blebbing, cellular fragmentation into membrane-bound bodies, phagocytosis of the dying cell, and absence of an ensuing

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inflammatory response.[1,2] Defects in apoptosis are essential in cancer pathogenesis, allowing cells to overcome nutrient deprivation, absence of growth-stimulating signals, presence of growth inhibitory signals, surrounding tissue barriers, hypoxia, oxidative stresses, and host immune responses.[3,4] Cancer cells survive beyond their physiologically intended lifespan and accumulate genetic alterations that increase cell proliferation, angiogenesis, invasiveness, and interfere with differentiation.[3,4] Of primary importance, apoptotic defects are required to complement proto-oncogene activation (e.g., Cyclin D1, E1A, Myc).[2,5]

Apoptotic cell death results from the activation of a subset of normally catalytically inactive intracellular cystein aspartyl-specific proteases, termed caspases.[6] In humans, at least 7 out of the known 11-12 caspases are involved in cell death.[3,6] These proteases cleave various cellular targets to induce apoptosis; two canonical signaling pathways for caspase activation are the death receptor and mitochondrial cascades (Fig. 1).

In the death receptor, or "extrinsic" apoptosis pathway, extracellular "death ligands" (e.g., FasL) activate a family of cell surface "death receptors" (e.g., Fas).[2,3,7] FasL-Fas binding results in receptor oligomerization and recruitment of Fas-associated protein with death domain (FADD). FADD oligomerization then recruits the procaspase-8 to this complex; proximity-induced dimerization and autoproteolytic cleavage occurs, producing active caspase-8. This "initiator" caspase can then cleave and activate downstream caspases, such as the "effector" caspase-3, which induces apoptosis by cleaving multiple targets.

Mitochondrial-mediated "intrinsic" apoptosis is induced by stimuli such as growth factor deprivation, oxidants, Ca^{2+} overload, oncogene activation, DNA damaging agents, and microtubule-attacking drugs.[8] The interactions and balance between pro-apoptotic (e.g., Bim, Puma, Noxa, Bax, Bak) and anti-apoptotic (e.g., Bcl-2, Bcl-x_L, Bcl-B) members of the Bcl-2 family (n = 24 in humans), located at the surface of the mitochondria, determines whether the cell lives or dies.[9] An increase in the activation of pro-apoptotic Bcl-2 family proteins, or a decrease in activation of anti-apoptotic Bcl-2 family proteins results in Bax and/or Bak activation. Bax or Bak can in turn, induce the release of several proteins from the mitochondrial inter-membrane space; these proteins include (1) inhibitor of apoptosis (IAP) inhibitors (which inhibit caspase-inhibitors); (2) nuclear degraders; and (3) cytochrome c.[1,2,9,10] Cytochrome c and Apaf-1 assemble with procaspase-9 in a complex known as the "apoptosome" to induce caspase function, leading to the cleavage of caspase-3.

There is cross talk between the death receptor and mitochondrial-mediated apoptosis pathways. For example, caspase-8 can cleave Bid, a pro-apoptotic Bcl-2 family member, to generate truncated Bid (tBid), leading to mitochondrial-mediated apoptosis.[2,9] Although not within the scope of this book chapter, many other proteins and organelles can induce apoptosis.[11] Herein, we will discuss the dysregulation of apoptotsis in NPC and the logical therapeutic targeting of this process.[11]

APOPTOSIS IN NPC

The past decade has seen the accumulation of a large amount of data elucidating mechanisms by which NPC cells might acquire resistance to apoptosis. For simplification, we will first focus on genes that are directly involved in the apoptotic pathway, and organize our discussion based on the primary method of discovery (see Fig. 2 and Table 1 for a summary).

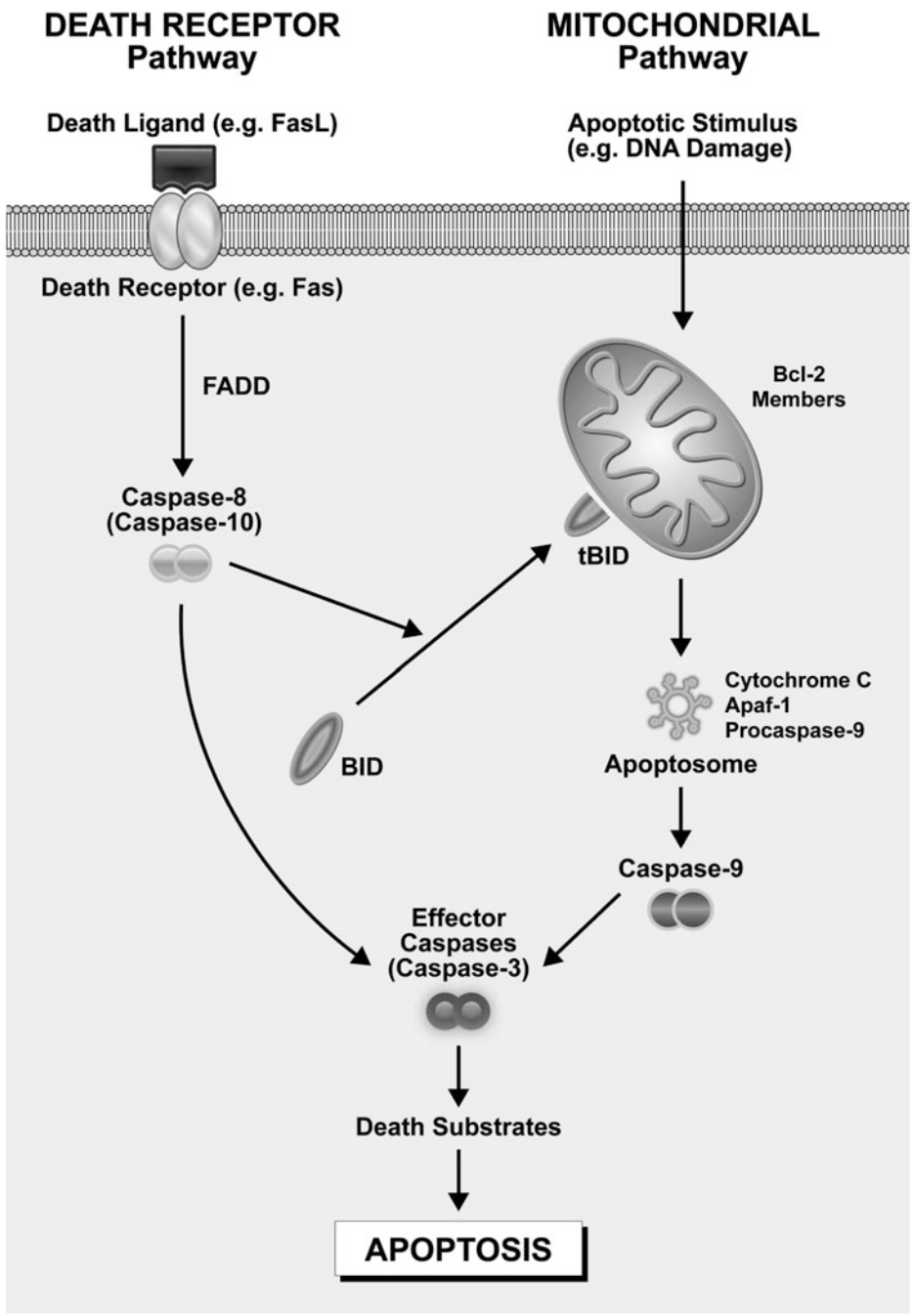

Figure 1. Death receptor and mitochondrial apoptosis pathways. Apoptosis can be activated by either the death receptor (extrinsic), or the mitochondrial (intrinsic) pathways. In both pathways, initiator caspases are activated, which in turn, activate effector caspases.

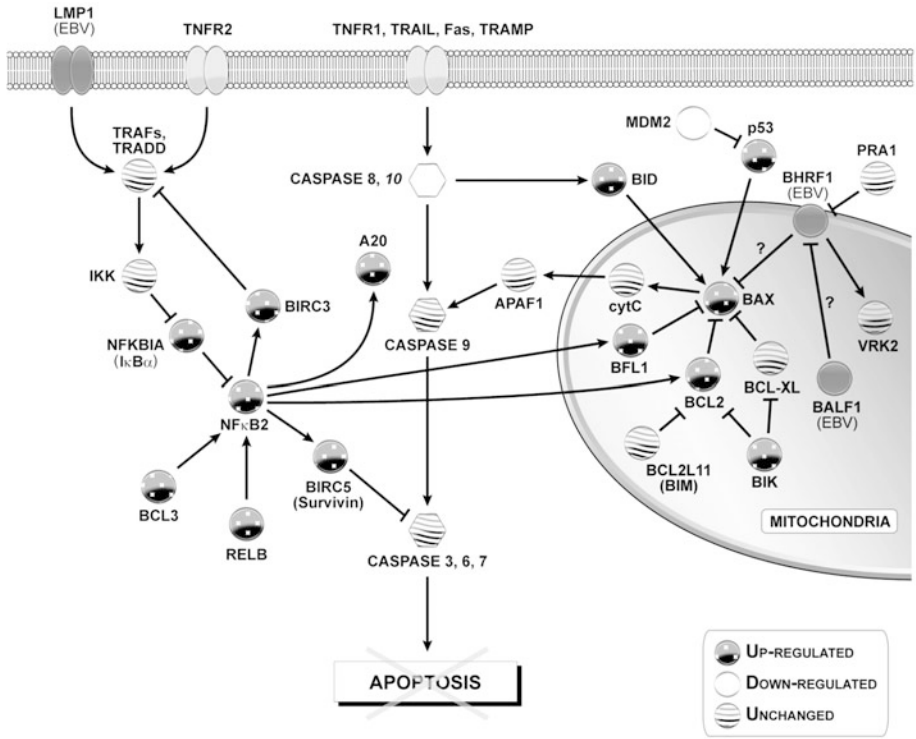

Figure 2. Schematic of the gene network leading to apoptosis resistance in NPC. This schematic was generated by integrating other groups' data into our micro-array results. Genes in solid grey are coded by EBV. Transcriptional activation and inhibition are indicated by arrows and T-lines, respectively.

Table 1. Summary of NPC associated apoptotic genes

Genes	Chromosomal Location	Aberrations in NPC	References
p53	17p13.1	Over-expression	13,27-29
p21	6p21.2	Over-expression	35-37
bcl-2	18q21.3	Over-expression	12-14
bcl-3	19q13.31	Ectopic expression	34,35
bcl-X	Xq28	Over-expression	26,42
DAPK	9q34.1	Inactivation by promoter hypermethylation	32,46,47
RASSF1A	3p21.3	Mutation, Inactivation by promoter hypermethylation	32,42,43
Survivin	17q25	Over-expression	13,17,18
c-Myc	8q24.12-24.13	Over-expression; amplification	12
Fas-L	1q23	Over-expression	20-22
NFKB2 (p100/p52)	10q24.32	Over-expression	26,40
TRAF1	9q33.2	Ectopic expression	65,66

Immunohistochemistry Data

Bcl-2 over-expression, as detected by immunohistochemistry (IHC), occurs in 61-89% of NPC tumors.[12-15] Both precancerous lesions and invasive NPC over-express Bcl-2, possibly suggesting that this alteration occurs early in NP tumorigenesis.[16] Interestingly, Bcl-2 is often co-expressed with Bcl-x_L in NPC; such a phenomenon is rarely observed in squamous cell carcinomas of the head and neck region.[15] Bcl-2 and c-Myc are co-expressed in 57-61% of NPC samples,[12] which in turn, is significantly associated with a higher risk of disease recurrence, and reduced survival.[12]

The IAP-family member survivin is also over-expressed in NPC (Fig. 2, Table 1).[13,17,18] Using IHC and Western blot analysis, our laboratory detected over-expression of both nuclear and cytoplasmic survivin in primary NPC samples.[13] There was an optimal level of survivin associated with clinical outcome, in that tumours with an intermediate level of survivin expression achieved the best overall survival.[13] In a separate study, patients with low survivin expression had better overall, disease-free, and distant metastasis-free survival.[18] The variation in results may be due to the complex roles of survivin in apoptosis and mitotic regulation, many of which depend on subcellular localization.[19]

A critical component of the extrinsic apoptosis pathway, FasL, is over-expressed in 32-75% of NPC samples[20-22] and associated with poor survival.[23] This apparently counter-intuitive observation might be attributed to a number of factors. FasL expression may: (1) down-regulate immune surveillance or destruction;[20,24] (2) merely indicate tumors with significantly aberrant apoptotic signaling;[20] or (3) be counterbalanced by expression of CD40, an inhibitor of FasL-mediated apoptosis that is expressed by malignant NPC cells and whose ligand CD40-L (CD 154) is expressed by tumour-infiltrating lymphocytes.[24]

Other genes that play a critical role in influencing NPC tumorigenesis and apoptosis resistance include p53 and NF-κB. p53 gene mutations exist in approximately 50% of human cancers.[25] In NPC, however, p53 mutations are extremely rare. In fact, p53 protein over-expression has been frequently observed in NPC by IHC, affecting 30-95% of such tumors.[13,26-29] Moreover, increased frequency of p53 over-expression is associated with more advanced NPC stages.[30] A recent tissue array study of 148 NPC samples and 164 adjacent noncancerous tissues reported abnormal p53 expression in histologically normal nasopharyngeal tissues, suggesting that this alteration occurs early in tumorigenesis.[31] Functional disruption of the p53 pathway may result from the inactivation of p14/ARF, thereby facilitating ubiquitination of p53.[32] Alternatively, over-expression of truncated deltaNp63, a p53 homolog, may block p53-mediated transactivation through a dominant-negative mechanism.[33] P53 induces the expression of Waf1/Cip1 (p21), which causes growth arrest.[34] As expected, NPCs with p53 over-expression typically also express p21, suggesting at least partial p53-functionalty.[35-37] Alterations in the apoptotic pathway may thus allow cells to overcome the pro-apoptotic effects of p53 overexpression.

NFκB pathway alteration is a particularly critical event in EBV-associated tumorigenesis.[38,39] EBV activates NFκB when normal epithelial cells are infected with EBV.[40] In NPC, the mechanisms of NFκB activation are complex, and are mediated, at least in part, by EBV LMP1. Over-expression of the NFκB p50 subunit may lead to NPC oncogenesis and disease progression *via* target gene (e.g., *EGFR*) transcription.[41]

Epigenetic Data

Cancer gene promoter hypermethylation is frequently observed in NPC. One of the most common epigenetic alterations is *RASSF1A* inactivation. This gene is also commonly deleted in NPC.[42] Mutations or *RASSF1A* promoter hypermethylation is reported in 10-35%, and 67-84% of NPC cases, respectively, resulting in its transcriptional silencing.[32,42,43] *RASSF1A* has been implicated in apoptotic signaling by functioning as a negative Ras effector.[44,45] Promoter hypermethylation of another apoptosis related gene, DAP-kinase, was reported in 26-76% of NPC using methylation-specific PCR analyses.[32,46,47] This protein is a mediator of apoptosis; hence its loss will result in apoptosis resistance.[48] Investigation of the methylation profile of DAPK, *RASSF1A* and p16 (yet another NPC tumor suppressor gene) have been evaluated in patients' biological samples, and were demonstrated as potentially effective NPC progression markers.[47,49]

Micro-Array Data

Several NPC gene expression studies have been performed, although only a few have published extensive gene lists from which transcriptional network models for apoptosis-related genes can be built.[50-56] Our global micro-array and pathway modeling work suggest that over-expression of NFκB2 plays a central role in apoptosis resistance (Fig. 2).[26] In particular, over-expression of NFκB2, NFκB transcriptional cofactors (e.g., RelB and Bcl3), and NFκB transcriptional targets (e.g., Bcl-2, Bfl-1, Birc3, Birc5 (survivin) and A20) were identified (Fig. 2). These data are in agreement with the aforementioned IHC studies, verifying over-expression of anti-apoptotic proteins.

As in many human malignancies, NPC cells over-express both pro-apoptotic (Bax, Bid and p53) and anti-apoptotic genes (Bfl1 and Bcl-2), resulting in significant and complex dysregulation of apoptosis (Table 1, Fig. 2). In particular, NPC mitochondrial-mediated apoptosis is inhibited through an unstable balance of proteins, which may support the therapeutic targeting of this pathway.

Because of the differences in microarray platforms (and hence genes studied), integration of other groups' data into the proposed model is difficult. Nevertheless, other micro-array studies indicate down-regulation of caspase-10 and MDM2 (a p53 inhibitor) as key mechanisms of apoptosis resistance (Figs. 1 and 2).[50]

EBV and Apoptosis

EBV plays an important role in the establishment and progression of NPC by promoting cellular proliferation and apoptosis resistance. Two EBV proteins often interfere with the execution of apoptosis: Latent membrane protein 1 (LMP1) and BHRF1 (Fig. 2). LMP1 is a functional homolog of CD40, a member of the tumor necrosis factor receptor (TNFR) family. The intracellular carboxy terminus of LMP1 contains two domains (CTAR 1 and 2), which interact with the TNFR-associated factors and Death Domain proteins (TRAFs and TRADD), resulting in the activation of NFκB.[57] LMP1 is constitutively activated, and blocks apoptosis in Burkitt's lymphoma and B cells.[58,59] In epithelial systems however, LMP1 plays a more complex role in that it can both promote and inhibit apoptosis, depending on the specific epithelial cell line, and the apoptotic stimulus.[60-62] The presence

of LMP1 is associated with up-regulation of the anti-apoptotic A20, survivin and bcl-2 proteins in a variety of epithelial cells[14,17,60] (Fig. 2).

EBV-infection also modifies TRAF1 (EBI6), another regulatory protein involved in apoptosis.[63-65] TRAF1 is a negative regulator of the death receptor pathway, but it contains a LEVD motif that can be cleaved by caspase-8, resulting in N- (1-163) and C-terminal (164-416) fragments.[66] The latter C-terminal fragment induces apoptosis, mediated through inhibition of NFκB activation.[66] TRAF1 cleavage occurs in NPC cells treated with Fas-agonists or chemotherapeutic agents,[64] suggesting that this cleavage might serve as an early indicator for therapeutic induction of apoptosis.[64]

EBV possesses two complex Bcl-2 homologues, BHRF1 and BALF1. BHRF1 is expressed early during the EBV lytic cycle,[67] and is structurally and functionally homologous to the anti-apoptotic Bcl-2.[68] Expression of BHRF1 can confer apoptosis resistance to serum-deprived Burkitt's lymphoma cells,[69] genotoxic drug-treated fibroblasts,[70,71] and serum deprived/Fas activated/TNF-α-treated epithelial cells.[72,73] Interestingly, BHRF1 may behave differently than Bcl-2,[74] and may have a role in PRA1 (prenylated rab acceptor 1) and vaccinia virus B1R kinase-related kinase 2 (VRK2) activity (Fig. 2).[75,76] Because BHRF1 expression takes place during the lytic phase, it is not always detected in EBV-associated tumors. It has therefore been suggested that BHRF1 plays an early role in oncogenic development.[77] In contrast, BALF1, which counteracts BHRF1 function, is expressed in 80% of NPC biopsies, at least at the transcriptional level. BALF1 does not inhibit human anti-apoptotic Bcl-2 genes such as Bcl-x_L, and hence does not clearly function as a pro-apoptotic gene.[78,79]

TARGETING APOPTOSIS IN NPC

Several apoptosis-promoting therapeutic strategies have been tested in NPC, targeting: (1) the mitochondrial pathway; (2) the receptor pathway; (3) EBV genes; or (4) unknown/ indirect apoptosis targets.

Targeting the Mitochondrial Pathway

Proteins of the Bcl-2 family possess one or more Bcl-2 homology (BH) domains: BH1, BH2, BH3, or BH4. Pro-apoptotic Bcl-2 family members are either multidomain BH123 (containing BH1, BH2, and BH3 domains) or BH3-only proteins.[8] Anti-apoptotic Bcl-2 family members, on the other hand, typically contain all four BH domains. Pro-apoptotic BH3-only proteins (e.g., Bim) function as the initiators of mitochondrial apoptosis, anti-apoptotic Bcl-2 proteins function as modulators (inhibiting the process; e.g., Bcl-2), and multidomain BH123 proteins function as the output (inducing the release of cytochrome c and other molecules from the mitochondrial intermembrane space; e.g., Bax, Bak).[3,8,9,80] Therapeutic targeting of the mitochondrial pathway in NPC has revolved around over-expressing BH3-only proteins, over-expressing pro-apoptotic multi-domain proteins, or inhibiting anti-apoptotic Bcl-2 proteins.

Many pro-apoptotic Bcl-2 proteins have been utilized for gene therapy. In NPC, tumor-specific apoptosis was achieved by incorporating an EBV-specific promoter (OriP system) with the highly pro-apoptotic Bim_S in an adenoviral gene therapy vector.[81,82] This vector was able to abolish EBV-positive NPC xenograft tumor formation in 7/9 mice

(followed for 100 d).[82] In combination with radiation therapy, intra-tumoral injections of the vector were able to significantly increase mouse survival time when compared to those treated with radiation therapy alone. In histological and biochemical toxicity assays, only minor liver toxicity was observed.[82] Less extensive therapeutic studies have been performed using *Bax*. Lipid-based transfection of *Bax* into HNE1 cells increased apoptosis, leading to a consequent effect on tumour growth delay.[83]

Bcl-2 has been targeted in several therapeutic NPC studies. A phosphorothioate antisense molecule targeting the first six codons of human Bcl-2 (similar to G3139, oblimersen sodium, Genasense; Genta Incorporated, Berkeley Heights, New Jersey) significantly down-regulated Bcl-2 in C666-1 cells.[84] This antisense molecule displayed single-agent efficacy in xenograft tumors; a more-than-additive interaction was observed when combined with radiation therapy.[84] In a separate study, the combination of Bcl-2 antisense and cisplatin was able to significantly inhibit the growth of established C666-1 xenograft tumors, curing 69% of the mice compared to 0% for mice treated with either agent alone.[85]

Many studies have explored the therapeutic potential of activating apoptosis through adenoviral vectors engineered to over-express p53 (adv.p53).[86-92] Our in vitro studies demonstrated that adv.p53 was effectively cytotoxic in CNE-1 and CNE-2Z cells (both of which express a mutated form of p53).[87] This cytotoxic effect was mediated by increased apoptosis,[90] and was independent of EBV status since this strategy was equally efficacious in either EBV-negative[87,90] or positive NPC[88] models. We also confirmed that over-expression of p53 interacted with ionizing RT in a more-than-additive manner.[90] In contrast, only an additive effect was observed when p53 over-expression was combined with hyperthermia or cisplatin.[89,91,92]

Targeting the Receptor Pathway

Fas and FasL are extensively expressed in primary NPC,[24,27,93] and the Fas-mediated apoptotic pathway is often intact and capable of inducing apoptosis in NPC cells.[93,94] We investigated the possibility of exploiting this pathway to induce apoptosis in NPC cells by generating an adenovirus expressing a noncleavable form of *FasL* (*ncFasL*) under the control of the EBV-specific OriP promoter (ad5oriP.*ncFasL*).[95] The ncFasL minimizes both FasL cleavage and the resulting soluble-FasL-induced hepatotoxicity,[96,97] without compromising biological activity.[98] We observed a significant induction of apoptosis by ad5oriP.*ncFasL* in the EBV-positive NPC cell line C666-1.[95] C666-1 ex vivo infection with adv.oriP.*ncFasL* completely prevented tumor formation in SCID mice followed for up to 100 days. Regression of established nasopharyngeal xenograft tumors was also observed in combination with radiation therapy after intra-tumoural delivery of adv.oriP.*ncFasl*.

Numerous studies suggest survivin as a potential target for NPC therapy.[13,18,26] Inhibition of survivin in NPC cells, either alone or combined with RT, significantly sensitizes cells to apoptosis.[26,99]

Targeting EBV Genes

The presence of EBV in NPC cells represents a potential tumour-selective therapeutic target. Although several EBV genes have been targeted, the discussion here will focus only on those genes directly involved with apoptosis. To date, there have been no reports demonstrating a therapeutic benefit from LMP1 down-regulation

in NPC. However, stable expression of a siRNA against LMP1 induced significant apoptosis in an EBV-positive marmoset lymphoblastoid cell line (B95.8).[100,101] In our experience, the same observations could not be recapitulated in NPC, wherein several different siRNA sequences targeting LMP1 in C666-1 cells failed to demonstrate any reduction in cell survival (unpublished data).

Other Approaches

Recently, phenotype-driven screening approaches have revealed novel potential NPC therapeutics. Our lab screened the 1120-compound Prestwick (Prestwick Chemical Inc., Washington, DC) and the 1280-compound LOPAC1280 (Sigma-Aldrich Corporation, Saint Louis, Missouri) chemical libraries for agents with preferential cytotoxicity for FaDu (human hypopharyngeal squamous cancer), and C666-1 cells, while impacting minimally on NIH/3T3 (untransformed mouse embryonic), and GM05757 (primary normal human) fibroblasts.[102] Benzethonium chloride and alexidine dihydrochloride were identified to induce cancer-specific apoptosis.[102,103] Although the molecular targets of these compounds have yet to be elucidated, such screens represent powerful platforms for the identification of apoptosis-inducing anti-cancer agents.

Although not within the scope of this chapter, a number of molecularly-targeted therapies are currently undergoing clinical testing in NPC, including CDK, EGFR, c-MET, and HDAC inhibitors.[104] These agents do not directly target the apoptotic pathway, but they do increase cancer cell apoptosis.[105-109]

CONCLUSION AND FUTURE DIRECTIONS

Apoptosis is significantly dysregulated in NPC, rendering the disease particularly amenable to such therapeutic strategies. The potential for developing clinically relevant NPC therapeutics remains promising. Pro-apoptotic agents must be targeted to cancer cells to reduce toxicity, yet at the same time, be systemically deliverable in significant concentrations. In particular, effective inhibition of anti-apoptotic Bcl-2 family members is becoming more readily achieved with the advent of newer antisense chemistries, siRNA modifications, and potent small molecules such as ABT-737.[110-113] Hence, the efficacy of such agents, particularly in combination with current therapeutic modalities, definitely warrants future clinical evaluations.

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