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Hepatocellular Carcinoma

Methods and Protocols

Edited by

Nagy A. Habib

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Hepatocellular Carcinoma

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Humana Press  **Totowa, New Jersey**

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Totowa, New Jersey 07512

This publication is printed on acid-free paper. ∞
ANSI Z39.48-1984 (American Standards Institute) Permanence of Paper for Printed Library Materials.

Cover design by Patricia F. Cleary.
Cover photo: Figure 1A from Ch. 2, *Medical Management of Hepatocellular Carcinoma*, by S. M. Riordan and R. Williams.

Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1
Library of Congress Cataloging in Publication Data

Hepatocellular carcinoma: methods and protocols / edited by Nagy A. Habib.
p. ; cm. -- (Methods in molecular medicine ; 45)
Includes bibliographical references and index.
ISBN 0-89603-785-1 (alk. paper)
1. Liver--Cancer--Research--Methodology. I. Habib, Nagy A. II. Series.
[DNLM: 1. Carcinoma, Hepatocellular--etiology. 2. Carcinoma, Hepatocellular--genetics. 3. Carcinoma, Hepatocellular--therapy. 4. Gene Therapy--methods. WI 735 H5295 2000]
RC280.L5 H456 2000
616.99'436--dc21

99-052577

Preface

Hepatocellular Carcinoma: Methods and Protocols outlines the research methods applied in the laboratories and clinics of those scientists and clinicians interested in the understanding and clinical management of patients with hepatocellular carcinoma (HCC).

Part I, *The Clinical Problem*, has been contributed by two leading clinical groups who have identified and addressed problem areas related to the management of HCC patients. Various treatment modalities are discussed and emphasis is placed on the limitations they experienced.

Part II, *HCC Carcinogenesis*, reviews the main etiological factors related to hepatitis B and hepatitis C.

Part III, *Molecular and Biological Characteristics*, provides insight into the molecular changes associated with HCC, including tumor-suppressor genes, oncogenes, adhesion molecules, matrix metalloproteinase, and novel genes and markers.

Part IV, *HCC Gene Therapy*, addresses gene therapy approaches to treating hepatocellular carcinoma. It includes the use of various vectors, such as lipids, viruses such as adenoviruses and baculoviruses, and virus detection using electron microscopy assessment. The use of adenovirus with specific promotors, such as AFP, is also included. Preclinical and clinical data on the killing of cancer cells using tumor-suppressor genes, antisense to growth factors, immunogene therapy, or virus-directed enzyme prodrug therapy are addressed.

Part V, *Clinical Protocol for* p53 *Gene Therapy for Liver Tumors*, offers a future perspective on how to treat the failing liver, which is the principal source of mortality in these patients. It includes gene therapy approaches for use in patients suffering from hypoalbuminemia, abnormal clotting, low platelet count, and viral hepatitis DNA vaccinations.

There is no doubt that advances in molecular characterization, novel gene isolation, and gene therapy will improve the services we can provide to patients suffering from hepatocellular carcinoma and ultimately working toward its prevention.

Nagy A. Habib

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I

The Clinical Problem

1

Hepatocellular Carcinoma

The Clinical Problem

Valery Usatoff and Nagy A. Habib

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies, responsible for over one million deaths annually world wide. The causal relationship between HCC and cirrhosis is clear, with the majority of cases of HCC occurring in patients with cirrhotic liver disease. Survival of untreated individuals is poor. At this stage, surgical resection provides the only chance of cure, but it is not suitable for the majority of patients in whom the tumor pathology or the underlying liver disease makes surgery hazardous. For most patients, nonsurgical treatment is the only option and this has led to the popularization of various regional and systemic modalities. Tumor stage is a significant predictor of survival and screening high-risk groups allows detection of tumors at an earlier stage, thereby increasing the likelihood of effective treatment.

This chapter sets the clinical scene for the rest of this book by outlining the important issues in the management of HCC with particular focus on the limitations of the current treatment modalities.

2. Epidemiology

The incidence of HCC in Western countries is low, but it remains a serious health problem globally, causing an estimated 1,250,000 deaths every year worldwide ***(1)***. Western countries have an incidence of about 4 in 100,000, whereas areas of Africa and Asia have an incidence of up to 150 in 100,000 ***(2)***. In Zimbabwe and Ethiopia, it accounts for up to 50% of all malignancies, whereas in Europe

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

and North America, it accounts for less than 2%. These geographical variations closely reflect the influence of local risk factors. It is clear that cirrhosis is the prime risk factor for HCC with up to 90% of patients having liver cirrhosis ***(3)***, but hepatocarcinogenesis also depends on the underlying cause of cirrhosis with interplay from secondary risk factors. Patients at high risk of developing HCC are those with cirrhosis caused by viral hepatitis and hemochromatosis. Those at moderate risk have alcohol, α-1-antitrypsin deficiency, and autoimmune hepatitis-induced cirrhosis, whereas cirrhosis from Wilson's disease, primary biliary cirrhosis, and sclerosing cholangitits have a relatively low risk ***(4)***. Male sex and cigarette smoking provide secondary risk factors for HCC in patients with cirrhosis ***(5)***.

The incidence of HCC among patients with cirrhosis was found to be 12.5% over a 3 yr compared to 3.8% among patients with chronic active hepatitis without cirrhosis ***(5)***. Recent interest has turned to the group of patients without cirrhosis that develop HCC . Aflatoxin B1 and thorotrast (a contrast material) are two carcinogens that induce HCC in the noncirrhotic liver. The relationship between HCC and human steroids is disputable and the effect of these agents is probably very minor ***(6)***. It would appear that both hepatitis B (HBV) and hepatitis C (HBC) virus can cause HCC in the noncirrhotic liver, but the HCV-induced HCC is more likely to be in older patients with cirrhosis ***(7)***.

3. Pathology

The stepwise development of tumors has been well established and is usually a complex process involving at least three steps: initiation, promotion, and progression. Each step can only occur if there is a breakdown of a natural protective barrier and the oncogene is the tool by which the tumor breaks down these barriers ***(8)***. In colorectal cancer, Vogelstein defined the stepwise progression from hyperplasia to adenoma to carcinoma and concluded that the process involves activation of an oncogene, with the loss of several tumor suppressor genes ***(9)***. No single oncogene has been defined as a constant occurrence for HCC but several tumor suppressor genes do appear more commonly. Arakawa et al. ***(10)*** first suggested that HCC emerges in an adenomatous hyperplastic nodule. Differentiation between regenerative nodules and low-grade dysplastic nodules and then between high-grade dyplastic nodules and overt HCC is difficult. An increase in size correlates with malignancy, and benign nodules are rarely larger than 2 cm. As these nodules grow in size, there is a loss of normal histological architecture and the portal supply is replaced by newly formed arterial vasculature.

The most widely used staging system for HCC is the tumor, nodes, metastasis (TNM) staging system **(Fig. 1)** and is based on the size, number and distribution of the primary lesion and also on the presence of vascular invasion, lymph node involvement, and distant metastases.

1. The TNM Staging of HCC

Tx	Primary cannot be assessed
T_0	No evidence of primary tumor.
T_1	Solitary tumor, ≤ 2cm diameter, without vascular invasion
T_2	Solitary tumor, ≤2 cm, with vascular invasion or Multiple tumors, limited to one lobe all ≤ 2cm without vascular invasion or Solitary tumor, >2 cm, without vascular invasion
T_3	Solitary tumor, >2 cm, with vascular invasion or Multiple tumors, limited to one lobe, ≤2 cm, with vascular invasion or Multiple tumors, limited to one lobe, >2 cm, with or without vascular invasion
T_4	Multiple tumors involving more than one lobe or Any invasion of major branch of portal or hepatic vein

Nx	Cannot assess nodes	M_x	Cannot assess metastasis
N_0	No regional node involved.	M_0	No distant metastasis
N_1	Regional nodes involved	M_1	Distant metastasis

Stage I	$T_1N_0M_0$
Stage II	$T_2N_0M_0$
Stage III	$T_{1/2}N_1M_0$
	$T_3N_{0/1}M_0$
Stage IV_A	$T_4N_{0/1}M_0$
Stage IV_B	$T_{1\text{-}4}N_{0/1}M_1$

Fig.1. TNM staging of HCC, data from **ref. *17*.**

4. Natural History

The mean survival of patients with HCC from diagnosis is approx 3 mo ***(11)***. There is however, a wide range, and the survival of patients is closely related to

the stage of the tumor and to the extent of underlying liver disease. The simplest system of incorporating both tumor stage and liver impairment was put forward by Okuda et al. ***(12)***, which was based on the presence or absence of ascites, tumor volume >50% of liver, serum albumin < 30 g/L, and serum bilirubin >30 mg/L. Recently, several prognostic models have been put forward based on the complex multivariate analysis of untreated or ineffectively treated patients ***(13,14)***. Although these models do not easily lend themselves to clinical practice, they give a good estimate of the median survival from time of diagnosis to be in the range of 1 mo to 12 mo. Another, somewhat retrospective way of looking at prognosis is to categorize patients into resectable or nonresectable. Patients with resectable tumors who undergo adequate surgery have a survival of 20–30 mo. Those with nonresectable disease have a survival of 3–10 mo ***(15)***.

5. Diagnosis and Staging

Once the suspicion of HCC is raised by clinical symptoms, ultrasound (US) scanning or elevated α-fetoprotein (AFP) levels, the aim of further investigations is to confirm the diagnosis, stage the tumor, and assess the underlying liver disease. Percutaneous biopsy may lead to tumor dissemination along the needle tract and convert a favorable tumor to an inoperable one. Biopsy can be useful in certain situations, but only after liver resection or transplant have been excluded as treatment options. For HCCs <2 cm, the detection rate by computer tomography (CT) scanning is 72%, whereas it is 93% by magnetic resonance imaging (MRI) ***(16)***. Hepatic angiography is very useful in confirming the diagnosis of HCC because of the very particular vascular features the tumor exhibits. CT angiography/portography and delayed CT scanning after intra-arterial injection of lipiodol are also very sensitive and specific imaging modalities to confirm the diagnosis and detect small lesions. Staging the tumor depends on documenting the number, site, and size of the tumor(s) and their relationship to the major vessels ***(17)***. Before curative surgery can be considered, extrahepatic disease needs to be excluded and the common sites are local nodes, chest, and bone. Hepatic resection can only be considered in patients with adequate functional reserve and this can be estimated preoperatively by a variety of methods. These tests rely on measuring either the synthetic function of the liver, its excretion of certain metabolites, or on an estimate of the likely remaining liver volume after resection. However a common standard does not exist.

6. Screening

Certain characteristics of HCC make screening and early detection an attractive proposition: The at-risk population can be identified, resection of early stage tumors can be curative ***(18)***, HCC tends to grow slowly and stay confined to the liver ***(19)***,

and early detection is possible with US and AFP monitoring ***(20)***. However, the sensitivity and specificity of AFP is limited. Only 50–70% of patients with HCC have elevated levels of AFP ***(15)***. Only approximately one-third of patients with small HCCs (<5 cm) have a serum AFP above 200 ng/mL ***(21)***. At a cutoff point of 100 ng/mL, the sensitivity is 60% and the specificity is 95%. The assay becomes more useful if repeated tests show increasing levels ***(22)***.

Ultrasound is widely used for screening because it is noninvasive and inexpensive, but the cirrhotic background of the liver makes detection of small tumors difficult. In a group of patients in which 50% were ultimately transplanted for cirrhosis, Dodd et al. ***(23)*** reported a detection rate of HCC nodules of 45%. Groups that have performed repeated ultrasound examinations are able to achieve sensitivity and specificity levels of 90% for tumors larger than 1 cm ***(24)***. Screening with AFP measurements every 2 mo and US every 3 mo has been shown to significantly increase the detection rate of HCCs less than 3 cm in diameter and with less portal vein invasion. As a result, more patients were deemed resectable and the clinical value of this approach was evidenced by a significant improvement in survival ***(25)***. Despite this apparent success of early detection, no screening program has so far succeeded in demonstrating a cost-effective way of detecting curable HCC ***(26)***.

7. Treatment

Because only 20% of patients are considered suitable for hepatic resection at the time of diagnosis, based on tumor stage or underlying cirrhosis, the nonresectional modalities of treatment are an important part of the clinician's armamentarium. In the following sections, we will review the different treatment modalities commonly employed for resectable and nonresectable HCC.

7.1. Liver Resection

Hepatic resection has long been considered the only potentially curative treatment of HCC. However, as a result of advanced tumor stage and underlying cirrhosis, less than 20% of patients are eligible for resection at the time of presentation. A tumor diameter of less than 5 cm is often used as a cutoff for resection because of the increased risk of additional nodules and, consequently, incomplete resection. Newer imaging can more confidently exclude secondary nodules, and, hence, even large tumors that are truly solitary may still be suitable for successful resection despite their size ***(27)***. There is often a balance between the radicality of resection for cure and the conservation of liver parenchyma to avoid postoperative hepatic decompensation. As summarized in **Table 1**, published series report a 1-yr survival rate between 56% and 88% and a 5-yr survival rate between 28% and 59%. Operative mortality in these series varies from 2% to 16% but needs to be considered in relation to the number and degree of cirrhotic patients in the series. The operative mortality rates are

less than 3% for noncirrhotics and have been reported as high as 25% for cirrhotic patients ***(38,39)***. It has been put forward that patients with Child's A cirrhosis could safely undergo extensive resections, but this is proving to be an unreliable measure of preoperative liver function. More than half of these patients will develop hepatic decompensation after resection, and if persistant, it indicates a poor prognosis ***(40)***.

Patient selection can be further refined using various preoperative assessments. Recent studies have redefined the cut-off level of indocyanine green, (ICG) retention at which a major hepatectomy can be safely performed ***(41)*** and others have shown that significant portal hypertension (hepatic venous pressure gradient ≥ 10mm Hg) is an accurate predictor of persistent postoperative hepatic decompensation ***(40)***.

The main problem with liver resection for HCC is the high recurrence rate, which has a median survival rate of 1 yr ***(37)***. The incidence of recurrence is reported as between 20% and 64% within the first year, between 57% and 81% at 3 yr, and between 75% and 100% at 5 yr ***(33–35,42,43)***. The majority (80–90%) of patients that develop recurrence do so within the liver and only 10–20% have distant metastases. The pattern of recurrence is such that 12–26% occur at the resected margin, 40–50% away from the margin, and in 20–25% of cases there is widespread multinodular recurrence ***(18)***. This reflects the potential sources of recurrence, namely positive margins, undiagnosed multifocal disease, and ongoing malignant potential in the remaining cirrhotic liver. As a result, it is difficult to confidently classify a resection as curative. Even with the most favorable tumors, tumor-free survival at 5 yr ranges from 20–30% in Asian patients to nearly 0% in Western patients ***(33,36,43,44)***.

7.2. Liver Transplantation

In patients with HCC and cirrhosis, liver transplantation offers the prospect of treating the tumor and the underlying liver disease. This has the attraction of avoiding the morbidity of postoperative liver failure, eliminating the chance of further tumor occurrence in the remaining diseased liver, and preventing progression of portal hypertension. Early results were disappointing because the procedure was performed on patients with advanced disease, and the resulting high recurrence rate, consequent on immunosupression, led to poor long-term survival. This was in contrast to the much better results that were observed in patients who underwent liver transplantation for cirrhosis and were incidentally found to have HCC in the resected liver. It became clear that survival after liver transplantation for HCC was linked to tumor stage. Selby ***(45)*** details the 5-yr survival rates by TNM stage as follows: stage I—75%, stage II—68%; stage III—11%. If selection criteria are applied, then consistently reasonable results can be obtained.

Table 1
Survival After Surgical Resection of HCC

Authors	No. of Cases	Operative Mortality (%)	1-yr Survival	3-yr Survival	5-yr Survival
Tsuzuki et al. ***(28)***	119	9	80	47	39
Franco et al. ***(29)***	72	7	68	51	—
Gozzetti et al. ***(30)***	168	8	—	57	36
Gennari et al. ***(31)***	84	16	85	45	28
Nagasue et al. ***(32)***	229	11	—	53	29
Izumi et al. ***(33)***	104	7	88	65	59
Chen et al. ***(34)***	205	4	56	36	28
Lai et al. ***(35)***	194	12	n/a	44	35
Takenaka et al. ***(36)***	280	2	88	70	50
Farges et al. ***(37)***	226	8	82	59	39

The ranges of 1-, 3-, and 5-yr survivals achieved in recent series are 45–71%, 21–45%, and 20–45%, respectively **(Table 2)**.

Most centers restrict transplantation to patients with less than three nodules, with tumors less than 3 cm in diameter and with no vascular invasion, although the most useful prognostic criteria have not yet been identified. These sorts of selection criteria have led to a reduction in the recurrence rate and a long-term survival rate comparable to non-HCC patients. Earlier recurrence rates were as high as 65% ***(51)***, but in a recent study of 48 patients, the actuarial survival rate was 75% at 4 yr with a recurrence rate of only 17% ***(52)***.

Despite its apparent attractiveness, liver transplantation for HCC has several limitations. Organ shortage is probably the main factor leading to long waiting periods and inevitable progression of the disease to a less favorable tumor stage. The high risk of recurrent viral hepatitis and the potential for increased growth of residual/recurrent tumor in the setting of immunosupression also pose significant problems.

7.3. Transcatheter Arterial Chemoembolization

Hepatocellular carcinoma derives the majority of its blood supply from the hepatic artery, compared to the surrounding normal liver, which is mainly supplied by the portal vein. This difference has been used to advantage in selectively treating tumor nodules with various forms of embolization. Arterial blood flow can be interrupted by selective catheterization of the hepatic artery branch feeding the tumor and instillation of embolizing agents such as metallic coils, gelfoam, or starch. To avoid extensive hepatic necrosis, patency of the portal vein needs to be confirmed before this can be undertaken. Simple embo-

Table 2
Results of Liver Transplantation for HCC

Group	No. of Cases	Operative Mortality(%)	1-yr Survival	3-yr Survival	5-yr Survival
Yokoyama et al. ***(46)***	80	13	64	45	45
Ismail et al. ***(47)***	21	38	45	21	21
Pichlmayr et al. ***(48)***	87	20	55	30	20
Farmer et al. ***(49)***	44	16	63	30	30
Haug et al. ***(50)***	24	17	71	42	—

lization of the hepatic blood supply will result in ischemic necrosis of more than 80% of the tumor, in most patients ***(53)***. Where gelfoam is used rather than metallic coils, the segmental branch will recannalize and the procedure can be repeated at intervals of 6–12 wk. Embolization has been combined with a variety of chemotherapeutic agents and with lipiodol to potentially prolong the local concentration of these agents.

Minor morbidity is common after this procedure, occurring in nearly 90% of patients ***(54)*** and has been termed the postembolization syndrome. The symptoms consist of abdominal pain, nausea, and fever and usually resolve within a week. Other complications include cholecystits. Mortality rates of less than 2% can be expected in patients with normal hepatic function, but this increases markedly in patients with poor liver reserve, being 37% in Child's C patients ***(55)***.

Nonrandomized controlled studies have shown significant improvement in survival ***(55–57)***; however, this has not been supported by randomized controlled studies ***(58)***.

Other variations on the intra-arterial approach to treatment have been used, but no randomized controlled trials have yet shown advantage over other methods. Neocarzinostatin is a proteinaceous antibiotic with antitumor effect. The styrene–maleic acid form has enhanced cytotoxicity and other pharmacological advantages. Styrene–maleic acid neocarzinostatin (SMANCS) has been used effectively in patients with unresectable HCC ***(59)***, but no comparative data are yet available.

7.4. Percutaneous Ethanol Injection

Under ultrasound guidance, a fine needle can be introduced into the tumor within the liver and ethanol injected to cause coagulative necrosis. HCC is more sensitive to the effects of the percutaneous ethanol injection (PEI) because of the difference in density between the soft tumor and the cirrhotic liver. This

causes nearly complete destruction of the tumor with minimal injury to the surrounding liver. Although other agents (acetic acid, hot saline, and chemotherapeutic agents) have been tried, ethanol is the most widely used because it is readily available, inexpensive, well tolerated by patients, has low systemic toxicity, is effective, and can be used repeatedly. The extent of necrosis is closely related to the size of the tumor, with small nodules (<3 cm) usually completely destroyed while larger ones only partially destroyed ***(60)***. It is usually performed as an outpatient procedure twice a week using 3–5 mL of ethanol on each occasion. Small lesions can be treated with 3–6 sessions, whereas larger tumors have been treated with up to 15 sessions ***(61)***. The assessment of tumor destruction is difficult and usually relies on repeated imaging and measurement of AFP levels ***(62)***.

The patients most suited for this treatment are those with low-stage HCC (single tumor <5 cm or no more than three nodules, each <3 cm) and a liver function that limits resection ***(6)***. Portal vein invasion is not a contraindication ***(63)***, but patients with Child's C cirrhosis should be treated with caution, especially because PEI seems not to modify the otherwise rapidly fatal outcome in these patients ***(64)***. The most common complications with this technique are transient abdominal pain and fever. Severe complications such as hemorrhage or hepatic abscess are infrequent, occurring in 1.7% of cases ***(64)***. The 1-yr survival rate is more than 90% in many series, and for small tumors in cirrhotic patients, the 3-yr and 5-yr survival rates are 63% and 39%, respectively ***(65)***. Recurrence rates at 1 and 2 yr are 28.3% and 54%, respectively ***(61)***, although it has been suggested that the majority of the so-called recurrences actually represent new tumors ***(66)***. Larger tumors can be treated with PEI but with less effect, prognosis being related to the presence of cirrhosis, tumor size, and the number and the level of AFP ***(67)***.

There are no prospective randomized trials comparing PEI to surgery, but retrospective matched control studies suggest that both modalities have similar outcomes for single HCCs less than 3 cm. The operative mortality of resection is offset by the greater recurrence rates after PEI. Surgery probably provides a better chance of cure for solitary lesions greater than 3 cm by adequately treating the surrounding foci of microscopic tumor, but possesses a greater procedure-related risk than PEI in patients with Child's B cirrhosis ***(37)***.

7.5. Radiotherapy

The conventional approach of whole-liver irradiation is not effective. At the dose required to destroy the tumor, the surrounding liver also undergoes hepatitis and even failure in a cirrhotic liver. Modern three-dimensional beam-focusing methods can minimize beam scatter and deliver the required dose more specifically to the tumor only. This method of targeted radiotherapy has shown

a partial response rate of 64% and a 3-yr survival rate of 41%. It may provide palliative treatment for patients with larger tumors and good liver function but is not a recommended treatment for patients with Child's C cirrhosis ***(65)***. A third of the patients developed gastroduodenal bleeding and it seems that external beam treatment is only useful as palliative treatment for a very select group of patients. The recent development of proton irradiation may overcome some of the shortcomings of external beam therapy ***(68)***. It has the benefit of limiting irradiation of nontargeted areas, although is still hampered by our inability to accurately identify the full extent of the disease.

Specific tumor targeting by intra-arterial injection of radiotherapeutic compounds is practiced by several centers. Most rely on compounds such as iodine-131 radiolabeled lipiodol or yttrium-90 microspheres injected into the hepatic artery and then preferentially concentrated in the tumor tissue. Patients with significant arteriovenous shunting through the tumor are excluded as are those with extrahepatic disease. Yttrium-90 is a pure β-emitter and has a greater cytotoxic range than iodine-131, making it more suitable for larger tumors ***(62)***. In 71 patients treated with yttrium-90 microspheres, an overall tumor response rate of 89% measured in terms of changes in AFP levels was reported. The median survival was 9.4 mo and the treatment was well tolerated ***(69)***. As yet, there are no prospective studies comparing these new treatments to other modalities.

7.6. Chemotherapy

Many different drugs have been evaluated as systemic chemotherapy agents for the treatment of HCC. The results have been disappointing and there are probably several reasons for this. First, the tumor nodules have a slow doubling time that makes them relatively resistant. Second, this resistance is further enhanced by the expression of the multidrug resistance gene and there is a low hepatic extraction of chemotherapeutic agents. Finally, some of the treatments have significant morbidity and reduction in quality of life ***(63,70).*** The best combinations have a response rate less than 20% and a median survival of 6 mo, with fewer than 25% of patients alive at 1 yr ***(37)***.

Attempts to augment this response with intra-arterial instillation of chemotherapeutic agents have also been disappointing. Several groups have shown it to be more effective in terms of response rate, but there has been no demonstrable survival advantage ***(71,72)***. It is now widely agreed that chemotherapy, whether systemic or intra-arterial, has very little role to play in the treatment of HCC and its use should be restricted to clinical trials ***(62,63,73)***.

The presence of nuclear estrogen receptors in hepatocytes has lead to the use of tamoxifen as a treatment for HCC. A review of the efficay of tamoxifen ***(74)*** showed that three of the five randomized trials demonstrated a positive influence on survival, but this was not confirmed by the others. Further trials would seem warranted.

7.7. Thermotherapy

Local, *in situ* destruction of HCC is possible with thermotherapy. The appropriate probe is introduced into the lesion under ultrasound control and the local temperature is either lowered or raised to such a level as to produce local tissue destruction. Cryosurgery, using probes cooled with liquid nitrogen, has been used mainly for metastatic liver tumors but has also been proven safe for HCC. The ability to treat multiple lesions without unnecessary destruction of normal liver makes these techniques attractive. Cryosurgery has generally been performed during a laparotomy, which may be prolonged. It has its own complications such as liver "cracking" and bile leakage, along with hemorrhage, liver abscess, myoglobinuria, and renal failure. The largest series reports on the treatment of 87 patients ***(75)***. The 1-yr, 3-yr, and 5-yr survival rates were 60%, 32%, and 20%, respectively. For patients with tumors less than 5 cm, the 5-yr survival rate was 51%. Recent studies have shown that the technique can be performed using the laparoscopic ***(76)*** or percutaneous routes ***(77)***.

Hyperthermia can be delivered to the tumor with various modalities: microwave-generated heat ***(78)***, radio-frequency electrocautery ***(79)***, and laser-induced heat ***(80)***. Heat dissipation via nearby vessels can be a problem, reducing the efficacy of the treatment, but occlusion of the portal vein flow during treatment can significantly increase the size of the destruction area ***(81)***. This necessitates establishing a Pringle's manoeuvre, but this can also be accomplished during laparoscopic treatment, as was recently demonstrated in a porcine model ***(82)***. Hyperthermia has been used in metastatic liver tumors, but no data exist on its efficacy for HCC.

7.8. Combination Therapy

Multimodality treatment is not a new concept in the treatment of cancer, and in certain circumstances, there may be a role for it in the treatment of HCC. Initially unresectable tumors can occasionally be resected after multimodality treatment. Sitzmann and Abrams ***(83)*** reported on a group of patients undergoing resection of initially unresectable HCC after a combination of external beam radiotherapy, chemotherapy, and radiolabeled antiferritin antibody. This group had a 5-yr survival of 50% compared to 44% for the initially resectable group. Another group ***(84)*** treated 571 patients with unresectable HCC using a combination of therapies. The overall 5-yr survival rate was 28%. In a small group of these patients, the initial treatment allowed for subsequent resection to be performed. This group had a 5-yr survival of 59%. They concluded that it may be worthwhile reassessing patients after treatment for unresectable tumors.

When percutaneous alcohol injection is used after initial transcatheter arterial embolization, the results are often significantly better ***(65)***. Similarly, when external beam radiotherapy is applied after transcatheter arterial embo-

lization, a significantly increased survival can be observed *(65)*. There appears to be some benefit in pursuing combinations of regional therapies that have complementary effects to each other.

8. Review of Trials

Unfortunately retrospective studies form the basis for most of the comparisons between different treatment modalities. Farges and Belghiti *(37)* looked at several comparative studies *(54,58,67,85–87)* and came up with a series of conclusions. For single HCCs less than 2–3 cm, surgery and percutaneous ethanol injection probably achieve similar results, whereas for single tumors larger than 3 cm, surgery offers a better chance of cure. They also concluded that transcatheter arterial embolization is probably best suited for patients with large or multiple HCC.

Simonetti et al. *(88)* recently reviewed 37 randomized controlled trials evaluating the different effects of nonsurgical treatments for mainly unresectable HCC. Several interesting observations are made. They found no randomized studies of resection, transplantation, or alcoholization. The trials that looked at survival in the untreated control arms quoted an enormous variation in survival, with 12 mo survival varying from 60% to 0%, making it difficult to compare possible advantages in treatment outcomes. They found no evidence to support the ongoing use of chemotherapy, either systemic or in combination with embolization. Tamoxifen was the only drug that showed potential benefit in patients with unresectable and advanced HCC. There was no data to justify the widespread use of transarterial catheter embolization as adjuvant treatment. The overall conclusion was that the current treatment options made the out look for HCC rather gloomy.

9. Conclusion

Hepatocellular carcinoma is a global disease killing more than a million people each year. The prognosis without treatment is poor. Surgery offers the only real chance of cure, but the majority have unresectable disease because of tumor stage or liver cirrhosis. The remaining cirrhotic liver after surgery has ongoing precancerous potential and this is manifest in the high recurrence rates. Liver transplantation may overcome this problem but has very limited availability, leaving nonresectional therapies to provide the mainstay of treatment. We have reviewed a range of treatment modalities in this chapter and the old adage "whenever there is a long list of treatment options, it is likely that none of them is perfect" certainly holds true. Each modality has its limitations, whereas others such as systemic chemotherapy

have been proven to be of little value. The recent use of multimodality therapies seems to be encouraging, but randomized trials are lacking. The future will see a move from focusing on advanced disease to that of prevention, screening, and more innovative treatments.

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2

Medical Management of Hepatocellular Carcinoma

Stephen M. Riordan and Roger Williams

1. Introduction

The possibility of effective treatment of hepatocellular carcinoma (HCC), one of the most common cancers worldwide, largely depends on its detection at an early stage before symptoms develop. Screening patients with known cirrhosis, in whom the overall annual incidence of HCC development is of the order of 1–6% ***(1,2)***, has the potential to improve the detection rate of such asymptomatic tumors. Such an approach is limited, however, by the fact that cirrhosis is unrecognized prior to presentation with HCC in up to two-thirds of patients in areas with a high incidence of this tumor, such as Asia, and in nearly half of those from low-incidence areas, such as the United Kingdom ***(3,4)***. Furthermore, the sensitivity of commonly employed screening tools, such as the serum α-fetoprotein (AFP) level and hepatic ultrasonography, is suboptimal for detecting small tumors ***(5)***. In addition, HCC may arise in noncirrhotic patients with chronic hepatitis or carriage of hepatitis B virus in the absence of histological abnormality ***(6,7)***. The fibrolamellar variant, which does not produce AFP, also arises in an otherwise normal liver and would not, therefore, be detected by conventional screening programs. Consequently, most patients continue to present with large HCCs that are not amenable to either of the potentially curative surgical options of resection or orthotopic liver transplantation (OLT). Resection is also precluded when lesions, even if small, are sited in an anatomically unsuitable central position or if hepatic functional reserve is considered to be inadequate. In cirrhotic patients, this assessment may be based on a number of parameters, including the residual hepatic volume after planned resection as measured by computerized tomography scanning,

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

the indocyanine green and bromosulfthalein retention rates, uptake of technetium-99m–diethylenetriaminepentaacetic acid–galactosyl human serum albumin, the serum lecithin aminotransferase level, the Child's class and its individual components, and the hepatic venous pressure gradient ***(8–12)***. Of these, a raised preoperative serum bilirubin level and a preoperative hepatic venous pressure gradient > 10 mm Hg are especially important predictors of postoperative hepatic decompensation ***(10,11)***. Consideration of Child's class alone is inadequate for selecting patients for hepatic resection, as unresolved deterioration in hepatic function subsequently occurs in more than 50% of Child's A patients ***(10)***.

Treatment modalities that have been used in nonsurgical candidates include transcatheter arterial chemoembolization (TACE), percutaneous ethanol injection (PEI), a combination of TACE and PEI, systemic chemotherapy, and hormonal manipulation with antiestrogens, antiandrogens, luteinizing hormone-releasing hormone agonists, and the somatostatin analog, octreotide. Survival comparable to that following surgery has been reported on occasion. Experience with proton irradiation, targeted radiotherapy using ^{131}I-lipiodol, radio-frequency ablation, interstitial laser photocoagulation, microwave ablation, and cryotherapy is also accumulating. Randomized controlled data allowing a comparison of these medical treatments are limited. Modalities such as immunomodulation, gene therapy, and tumor vaccines are discussed elsewhere in this volume. Although an antitumor effect may be obtained, intervention may not necessarily modify the overall prognosis in Child's C patients, in whom survival is often determined by advanced cirrhosis and its complications rather than progression of the complicating HCC.

2. Transcatheter Arterial Chemoembolization

Transcatheter arterial chemoembolization (TACE) combines targeted chemotherapy with temporary hepatic arterial embolization and is a valid treatment option for patients with HCC confined to the liver, including large or centrally located tumors not amenable to other local treatments. Efficacy of TACE is improved by emulsifying the chemotherapeutic agent(s) with an iodized oil, such as lipiodol ***(13)***. The latter prolongs the contact time between anticancer drugs and tumor cells as a consequence of its selective retention by the tumor and by causing temporary sinusoidal embolization. Intra-arterial injection of particulate matter, such as gelfoam, is included in most regimens to further embolize tumor neovascularity. Some protocols additionally incorporate the injection of noradrenaline to constrict normal vasculature and thereby shunt the chemoembolization mixture into the tumor circulation. TACE is generally performed at 6–12 weekly intervals until tumor neovascularity is ablated. Main portal vein occlusion and sepsis are contraindications to TACE.

Many centers also exclude patients with Child's C cirrhosis in view of the possible risk of further hepatic decompensation consequent to transient ischemia of the nontumoros liver. The prevalence of this complication is reduced when gelfoam is not included in the TACE regimen, although the antitumor effect is less ***(14)***. Transient fever and right-upper-quadrant pain occur in most patients following the procedure. Uncommon untoward effects include liver abscess, renal failure, and neutropoenic sepsis.

Tumor ablation rates following repeated sessions of TACE are substantially higher for HCCs < 4 cm in diameter than for larger tumors, and multivariate analysis has identified tumor size, along with underlying liver function, as an important factor influencing survival following this form of treatment ***(15,16)***. In a nonrandomized study, Bronowicki et al. ***(17)*** found comparable 5-yr survival rates in patients with small, resectable HCCs treated with TACE, resection, or OLT. Furthermore, the probability of tumor recurrence and/or metastatic dissemination was lower after TACE than following surgery. Similar 1-yr survival to that attained with PEI has similarly been reported in a nonrandomized study ***(18)***. Prospective, randomized controlled studies comparing the survival rate following treatment of small HCCs with TACE to those obtained with resection, OLT, PEI, and other modalities discussed later are required. In practice, repeated TACE has predominantly been used to date in patients with large HCCs, not suitable for any of these other treatments. Uncontrolled studies have demonstrated 3-yr survival rates of 13–41% in this setting ***(19,20)***. However, two randomized controlled studies comparing TACE using a single chemotherapeutic agent with no treatment have not demonstrated a survival benefit ***(21,22)***, at least in part because of instances of treatment-related liver failure masking any possible survival benefit resulting from tumor ablation.

We recently reported our experience with serial, multiagent chemoembolization of HCC using cisplatin, doxorubicin, lipiodol, and gelfoam ***(23)***. Most patients were Child–Pugh class B or C and had large, inoperable tumors. Deterioration in Child–Pugh class due to ischemia of nontumoros liver was acceptably low and rarely led to unscheduled hospitalization. Varying degrees of control of tumor neovascularity occurred for a median of 390 d in over 97% of patients. Ablation of tumor neovascularity **(Fig. 1)** was attained in 100% of patients with HCC < 4 cm in diameter and over 33% of those with larger tumors. Significantly more sessions were required to ablate larger tumors. Tumor recurrence in the region of the original lesion was documented in 50% of patients after a median follow-up of 240 d, most often in those with larger initial tumors **(Table 1)**. Actuarial survival was 50% at 1 yr and 29% at 2 yr. No long-term survivor was Child–Pugh class C at the outset of treatment.

Limited available data suggests that preoperative TACE to reduce tumor bulk may have a role in improving the postoperative outcome in patients with

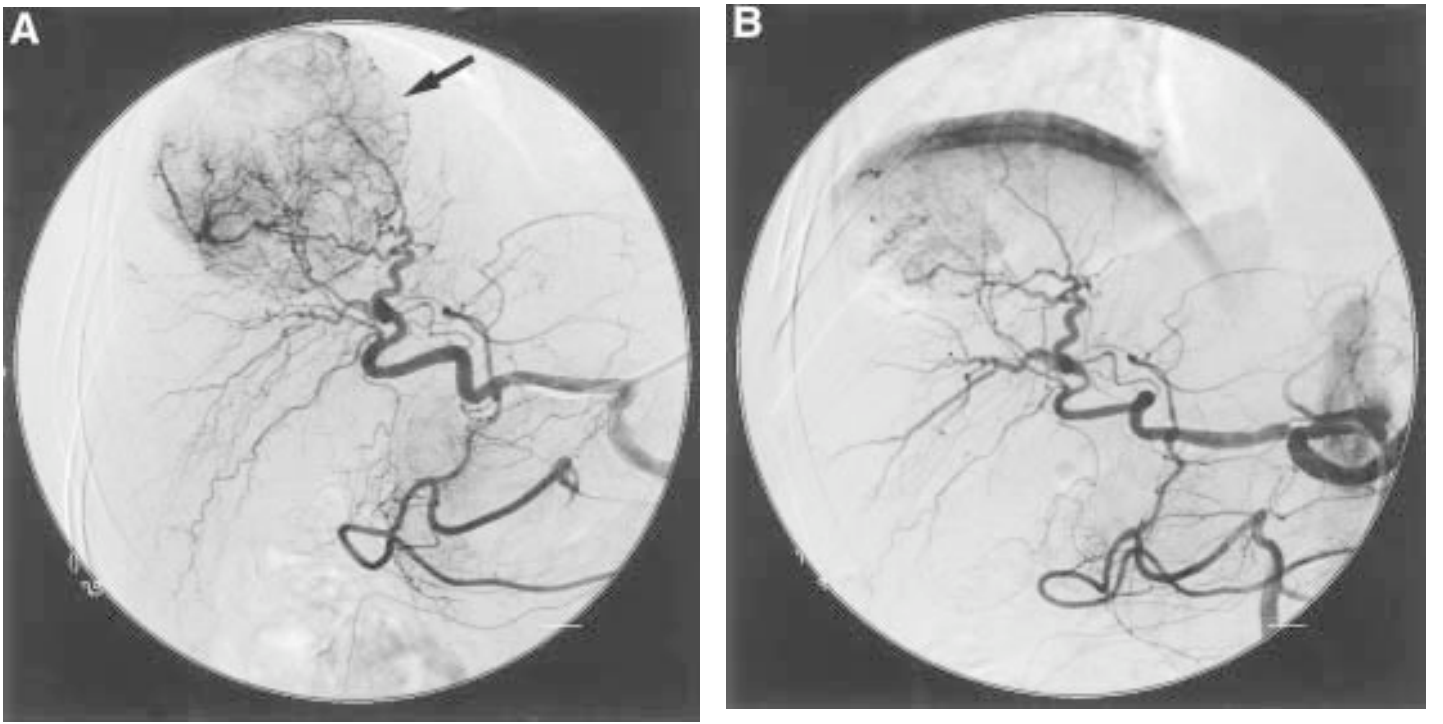

Fig. 1. Serial hepatic arteriography performed in a patient with a large hepatocellular carcinoma (HCC) before (a) and after (b) treatment with transcatheter arterial chemoembolization (TACE), demonstrating substantial reduction in tumor neovascularity (arrow) following treatment. (Reproduced with permission from **ref. *23***.)

Table 1
Rates of Ablation of Tumor Neovascularity with TACE and Recurrent HCC Development in Relation to Size of the Largest Initial HCC Focus

Mean Diameter of Largest HCC Focus (cm)	Ablation of Tumor Neovascularity (%)	Median (Range) TACE Sessions Required for Ablation of Tumor Neovascularity	HCC Recurrence (%)	Time to Detection of HCC Recurrence (d)
< 4	6/6 (100)	3.5 (2-6)	2/6 (33.3)	60, 160
4–7	4/12 (33.3)[a]	5 (4–6)	2/4 (50.0)	120, 360
≥ 8	6/16 (37.5)[a]	6 (4–6)[b]	4/6 (66.7)	90, 210, 240, 240

[a] $p < 0.02$ compared to mean diameter of largest HCC focus < 4 cm.
[b] $p < 0.05$ compared to mean diameter of largest HCC focus < 4 cm.
Source: Reproduced with permission from **ref. *23***.

HCCs considered borderline for resection, although not all experiences have been favorable *(24–26)*. There is some evidence to suggest that preoperative TACE may significantly prolong the interval between resection and HCC recurrence *(27)*. Literature concerning the possible efficacy of TACE as a means of reducing tumor size to fulfill suitability criteria for OLT is also limited. Although treatment does limit tumor progression in the majority of patients with small HCCs who are awaiting OLT *(28)*, any possible influence on post-OLT tumor recurrence and survival rates has not been adequately assessed.

3. Percutaneous Ethanol Injection

Percutaneous ethanol injection (PEI) of up to 10 mL of absolute alcohol under ultrasound guidance is appropriate in patients with a single HCC focus ≤ 5 cm in diameter or ≤ 3 tumor nodules, each ≤ 3 cm in size, especially if superficially located. The procedure is usually repeated one to three times weekly for several weeks until necrosis of the tumor, as evidenced by a lack of lesion enhancement on contrast-enhanced computerized tomography or magnetic resonance imaging. A single treatment using a larger volume of ethanol is also effective and generally well tolerated. Transient local pain sometimes requiring narcotic analgesia is the most common side effect, even when smaller volumes of ethanol are used. Other complications such as liver abscess, bile duct injury, hemoperitoneum, or chemically induced portal vein thrombosis occur in < 2% of patients. Rare instances of liver necrosis have also been reported *(29,30)*. The latter have been linked to the possible occlusion of hepatic arterial and portal venous supply resulting from vasculitis induced by ethanol extruded from the lesion *(31)*. As with percutaneous biopsy, needle tract seeding is another potential, though uncommon, complication of PEI *(32)*. Contraindications include ascites and uncorrectable coagulopathy. PEI is not effective against larger tumors, as the texture of the tumor parenchyma and the presence of septa prevent the homogeneous distribution of ethanol within the lesion. PEI is similarly not of value in patients with known extrahepatic dissemination.

Studies by Ebara et al. *(33)* and Livraghi et al. *(34)* in patients with favorable tumor characteristics have demonstrated 3-yr survival rates following PEI of up to 79%, depending on the underlying Child's classification. Survival was 0–25% in Child's class C patients **(Table 2)**. Isobe et al. *(35)* in a nonrandomized comparative study found significantly better survival in patients treated with PEI than in a nontreated control group. In addition, Castells et al. *(36)* and Kotoh et al. *(37)* have reported comparable cumulative survival and recurrence rates following PEI and hepatic resection in patients with small HCCs, despite less rigorous patient selection in the PEI group. In view of its lower associated morbidity and cost, PEI is consequently becoming increasingly used as an alternative to hepatic resection in patients with small, resectable HCCs for whom OLT is not available or

Table 2
Survival Rates Following PEI for HCC

HCC Characteristics	Child's Class	3-yr Survival (%)
≤ 3 foci, each ≤ 3 cm diameter (**ref. *33***)	A	72
	B	72
	C	25
Single focus, ≤ 5 cm diameter (**ref. *34***)	A	79
	B	63
	C	0
2–3 foci, each ≤ 3 cm diameter (**ref. *34***)	A	68
	B	59

otherwise contraindicated. However, patients remain predisposed to the development of new HCC foci in the preneoplastic cirrhotic liver. The latter occurs in approximately 50% of patients by 3 yr.

4. Combination TACE and PEI

Combined modality treatment with an initial session of TACE followed after 2 wk by a course of PEI has been proposed for large HCCs, especially when encapsulated, on the basis that complete necrosis of large lesions with TACE alone occurs in approximately 50% or less of cases and that prior TACE would disturb tumor parenchyma and disrupt septa such that ethanol is distributed more evenly throughout large lesions. Two randomized studies performed in patients with HCC > 3 cm in diameter found that tumor ablation rates were substantially higher after combined TACE and PEI treatment than with repeated sessions of TACE alone ***(38,39)*** **(Table 3)**. One-year survival was significantly better in the former group, ranging from 85% to 100% compared to 48–68% in those treated with TACE alone. A recent report of two cases of liver infarction following the PEI component of a combined TACE+PEI regimen, compared with no such instances in 205 patients treated with PEI alone, raises the possibility that this complication may be more prevalent with combination treatment, especially when styrene–maleic acid neocarzinostatin is used for TACE ***(40)***. Nonetheless, combined TACE and PEI should be considered the treatment of choice at present for patients with large, inoperable HCCs. Conversely, combined TACE and PEI does not improve the efficacy of PEI alone in the treatment of patients with smaller HCCs ***(41)***.

5. Irradiation

Conventional external beam radiotherapy is not only ineffective against HCC but also may precipitate liver failure as a consequence of radiation

Table 3
Tumor Ablation and Survival Rates Following Treatment with Transcatheter Arterial Chemoembolization Followed by a Course of Percutaneous Ethanol Injection (TACE + PEI) or Repeated Sessions of TACE alone for HCCs > 3 cm in Diameter

	HCC Ablation Rate (%)	
Authors (Ref.)	TACE	TACE + PEI
Bartolozzi et al. ***(38)***	52	85[a]
Tanaka et al. ***(39)***	20	83

[a]$p < 0.05$.

hepatitis. Proton therapy is a relatively new method by which a large amount of rediation can be focused on the lesion, limiting exposure of adjacent nontumoros liver. Matsuzaki et al. ***(42)*** applied this technology as monotherapy to 21 patients with HCC and documented at least a 50% reduction in tumor size in the majority of cases. Nonetheless, this therapy is currently limited to only a few centers. Preliminary clinical experience with targeted radiotherapy using intrahepatic arterial injection of lipiodol labeled with iodine^{-131}, a β- and γ-emitter that produces a local tumoricidal effect, is also available. In a prospective, randomized trial, tumor size at 2 mo remained static or was partially diminished in 68% of treated patients, comparable to that in those receiving lipiodol-based TACE with epirubicin ***(43).*** Actuarial survival rates at 6, 12, and 24 mo were also comparable in the two groups. Large-scale studies to determine the safety and efficacy of these irradiation techniques are awaited.

6. Thermal Modalities

Thermally mediated techniques such as radio-frequency ablation, interstitial laser coagulation, microwave therapy, and cryoablation each induce cell death by coagulative necrosis. Initial clinical experiences with these modalities are, in general, promising. Radio-frequency energy is delivered to the tumor by electrically insulated 14- to 17-gage needles introduced percutaneously under ultrasound guidance. Radiofrequency ablation of HCCs is achieved in a single session in 85–90% of cases when the tumor is smaller than 5 cm in diameter ***(44)***. The rate of local recurrence is less than 10%. Preliminary clinical data in small numbers of patients are also available for interstitial laser coagulation with neodymium–yttrium–aluminum–garnet. Bremer et al. ***(45)*** used this modality in two patients with HCCs no larger than 4 cm in diameter, with some evidence of stabilization of tumor size over follow-up of up to 11 mo. Photosensitization with protoporphyrin synthesized from administered δ-aminolevulinic acid followed by laser therapy has recently been shown to have an anti-HCC effect in an

experimental rodent model ***(46)***. Whether such therapy will be applicable to human HCC has not yet been addressed. Percutaneous microwave coagulation therapy is associated with tumor ablation or reduction in size in 70% of HCCs ≤ 3 cm in diameter and in 55% of larger lesions ***(47)***. Higher response rates have been documented in well-differentiated than in poorly differentiated HCCs. Sato et al. ***(48)*** found that this technique can effectively coagulate an area up to 6 cm in diameter in a single session. Use of multiple electrodes may increase applicability to patients with larger tumors. As with PEI, instances of tumor dissemination along the needle tract have been observed ***(47)***. Experience with cryotherapy for HCC is limited. In general, this form of therapy must be delivered by ultrasound guidance at laparotomy, thereby limiting its applicability in patients who cannot tolerate general anesthesia because of hepatic decompensation or concurrent medical problems.

7. Hormonal Manipulation

A number of randomized controlled trials have investigated the possible role of treatment with the antiestrogen drug, tamoxifen. Earlier studies performed in relatively small numbers of patients and using varying doses up to 60 mg daily yielded conflicting results ***(49–52)***. An Italian study of a large, multicenter cohort of nearly 500 patients randomly allocated to receive 40 mg of tamoxifen daily or no hormonal treatment suggests that tamoxifen has no overall efficacy in prolonging survival in patients with HCC ***(53)***. Whether treatment confers any benefit in that subgroup of HCC patients whose tumors express high levels of normally functioning estrogen receptors has not been investigated. Use of luteinizing hormone-releasing hormone analogs and the anti androgens flutamide and nilutamide has no appreciable effect on survival, despite the resultant suppression of sex-hormone synthesis ***(54–56)*** Conversely, a recent study performed in a small number of patients with inoperable HCC found that treatment with the somatostatin analog octreotide was associated with improved survival ***(57)***. Further studies are required to confirm this finding and to determine the possible mechanism of antineoplastic action.

8. Systemic Chemotherapy

Systemic chemotherapy with a variety of agents, including doxorubicin, epirubicin, mitoxantrone, cisplatin, and etoposide, either alone or in combination, is often used in patients with HCC disseminated beyond the liver, although response rates are generally of the order of only 15%. Furthermore, the value of systemic chemotherapy has never been confirmed in controlled trials. Consequently, this form of treatment has only a limited role in the management of HCC. Recent interest has centered on the possible efficacy of a liposomal doxorubicin preparation preferentially retained by HCC cells and a multicenter trial is currently in progress.

9. Preventive Measures

As the rate of recurrent and, especially, second primary HCC development is high in non-OLT recipients in whom the cirrhotic liver remains *in situ*, despite the apparently successful treatment of the initial tumor, several groups have investigated the possibility of secondary chemoprevention in this group. Muto et al. ***(58)*** developed a novel synthetic acyclic retinoid, polyprenoic acid, which binds to the cellular retinoic-binding protein and has been shown to suppress both chemically induced and spontaneous HCC development in rodents ***(59,60)***. A placebo-controlled, prospective analysis of 89 patients free of disease after successful treatment of HCC with resection or PEI, predominantly with chronic hepatitis C virus (HCV) infection, demonstrated a threefold reduction in the incidence of recurrent or new tumor development at a median follow-up of 38 mo in the group randomly allocated to receive the retinoid ***(58)***. The mechanism of action against human hepatocarcinogenesis in vivo remains to be clarified, although the drug has been shown to induce apoptosis in hepatoma cell lines by blocking transforming growth factor-α activity ***(61)***. A retrospective study suggests that treatment of patients with chronic HCV infection with interferon-α may also reduce the progression from cirrhosis to HCC by over sixfold ***(62)***. Prospective studies are required to confirm this observation. Patients with HCV-related cirrhosis should be encouraged to abstain from alcohol, as HCV and alcohol appear to act synergistically in promoting HCC development. In genetic hemochromatosis, the antineoplastic focus is on early iron chelation therapy in order to prevent the development of cirrhosis.

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II

HCC Carcinogenesis

3

Possible Involvement of the NS3 Protein of Hepatitis C Virus in Hepatocarcinogenesis

Its Interaction with the p53 Tumor Suppressor

Satoshi Ishido, Tsunenori Fujita, and Hak Hotta

1. Introduction

Hepatitis C virus (HCV), a member of the Flaviviridae family, is an enveloped virus, whose genome is single-stranded, positive-sense RNA of approximately 9.5 kb. The viral genome exhibits a considerable degree of sequence variation, based on which HCV is currently classified into at least 6 clades (previously called genotypes) and more than 60 subtypes ***(1,2)***. The HCV genome encodes a polyprotein consisting of about 3010–3033 amino acid residues. A number of studies have shown that this polyprotein is cleaved co-translationally and posttranslationally into mature viral proteins, which are arranged in the order NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH ***(3,4)***.

Hepatitis C virus causes either acute or chronic persistent infection, with the latter potentially leading to liver cirrhosis and hepatocellular carcinoma (HCC) ***(5,6)***. The viral genome and antigens have been detected in affected liver cells ***(7)***. The degeneration of the infected hepatocytes is caused either directly by cytopathic effect of the virus or indirectly by immune responses of the host, mainly through the function of cytotoxic T-lymphocytes (CTL). Continual occurrence of degeneration followed by regeneration of hepatocytes, together with fibrotic changes of the liver tissues, would result in liver cirrhosis and may possibly be responsible for development of HCC. On the other hand, a particular HCV protein(s), in concert with the other HCV proteins, may directly be involved in hepatocarcinogenesis by the virus. For example, an amino-ter-

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

minal portion of NS3 ***(8)*** and the core protein of HCV ***(9,10)*** have been reported to be involved in malignant transformation of the host cell. However, the exact mechanism by which HCV-infected hepatocytes become HCC still remains to be clarified.

Apoptosis of virus-infected cells, either mediated by a viral protein(s) or by CTL recognizing the viral antigens, has currently been considered as a mechanism of clearance of the virus from the host ***(11)***. Suppression of apoptosis, on the other hand, is accordingly thought to be a major mechanism of viral persistence in the infected cell, and in the case of infection with tumor viruses, it would be a crucial step toward malignant transformation of the cell. For example, Epstein-Barr virus latent gene products ***(12,13)***, adenovirus E1B 19-kDa protein ***(14)***, and human papillomavirus E6 protein ***(15)*** have been reported to inhibit apoptosis. These findings led us to a hypothesis that an HCV protein(s) has antiapoptotic activity to establish persistent infection through the interaction with certain tumor-suppressor protein(s), which might be an important step toward the development of HCC. In this chapter, we summarize our observations ***(16–19)*** that suggest possible involvement of NS3 in hepatocarcinogenesis.

Actinomycin D has been known to be a potent inducer of apoptosis through the induction of p53 ***(20–22)***. We demonstrated that NS3-expressing cells were more resistant to actinomycin D-induced apoptosis than the control cells. We also observed that induction of p53 expression by actinomycin D treatment was weaker in the NS3-expressing cells than in the controls. We do not know, at present, whether the decrease in the amount of p53 was the result of decreased transcription/translation or increased degradation of p53. If the latter is the case, NS3 might functionally resemble human papillomavirus E6, which directs specific degradation of p53 ***(15,23)***. We then examined the possible interaction between NS3 and p53. Immunofluorescence analysis revealed that, upon coexpression, NS3 and wild-type (wt)-p53 were colocalized in the nucleus. Moreover, immunoprecipitation analysis revealed that NS3 could form a complex with wt-p53. By deletion mutational analysis of NS3, an N-terminal portion of NS3 was shown to be important for the complex formation with wt-p53. As for wt-p53, its C-terminal portion (amino acids 301–393, or more specifically, 319–360) was shown to be important for the complex formation with NS3. Because the region between amino acids 319 and 360 has been known to be the oligomerization domain of wt-p53, it is not unreasonable to assume that NS3 interferes with wt-p53 oligomerization and, hence, its function. Indeed, by using a chloramphenicol acetyltransferase (CAT) reporter gene under the regulation of the wt-p53-responsive bax promoter, we

demonstrated that NS3 could inhibit the wt-p53-mediated transcriptional activation. Taken together, our results suggest the possibility that NS3 plays an important role in hepatocarcinogenesis through functional inactivation of wt-p53.

2. Materials

2.1. Plasmids

1. NS3 expression plasmids. A cDNA encoding a carboxy terminally truncated form of NS3 (NS3△C: amino acids 1027–1459) was obtained by polymerase chain reaction (PCR) using HCV-BK146 cDNA ***(24)*** as a template and a set of primers, NS3-S1 and NS3-R1 **(Table 1)**. The amplified fragment was subcloned into the unique *Eco*RI site of pH8 expression plasmid vector ***(25)*** and pBlueScript II SK- (Stratagene Cloning Systems, La Jolla, CA) to generate pHns3/1027–1459 and pBSns3/1027–1459, respectively. pBSns3/1027–1459 was digested with *Nco*I to remove two continuous *Nco*I internal fragments and then self-ligated to generate another plasmid, pBSns3/1201–1459, which mediates expression of amino terminally truncated NS3△C (NS3△N△C-1; amino acids 1201–1459). The region for the full-size NS3 (NS3F; amino acids 1027–1657) was amplified from HCV MKC1a cDNA (DNA Data Bank of Japan [DDBJ] accession number D45172) by using a set of primers, NS3-S1 and NS3-R2. The amplified fragment was digested with *Eco*RI and subcloned into pH8 and pBlueScript II SK- to generate pHns3/1027–1657 and pBSns3/1027–1657, respectively. To obtain pBSns3/1027–1245–FLAG, pBSns3/1027–1459 was digested with *Nde*I and *Bam*HI and ligated to an oligonucleotide encoding the peptide FLAG (DYKDDDDK). The FLAG peptide carries an antigenic epitope that can be specifically detected by anti-FLAG monoclonal antibody (M2, Kodak). **Figure 1a** is the schematic representation of the structure of the HCV genome and cDNA fragments used for construction of the expression plasmids.
2. wt-p53 expression plasmids. To express wt-p53, pCDM8VAarg/neo ***(26)*** was used. As a control, pCDM8/neo was used ***(26)***. A series of deletion mutants of wt-p53 were obtained as described below. The *Xho*I fragment of pCDM8VAarg/neo was ligated to *Xho*I-treated pBlueScript II SK- to generate pBS53/1-393 **(Fig. 1b)**. To obtain pBS53/40-393, the corresponding portion was amplified from pBS53/1-393 by using primer 53-S1 and 53-R1 **(Table 1)**. To obtain pBS53/1-360, pBS53/1-318, and pBS531-300, corresponding portions were each amplified from pBS53/1-393 by using sets of primers, 53-S2/53-R2, 53-S2/53-R3, and 53-S2/53-R4, respectively. These PCR products were digested with *Eco*RI and *Xho*I and inserted into the *Eco*RI and *Xho*I sites of pBlueScript II SK-.
3. The CAT reporter gene under the regulation of wt-p53-responsive bax promoter (pTM667-3) ***(27)*** was a kind gift from Dr. John C. Reed (The Burnham Institute, La Jolla, CA).

Table 1
Nucleotide Sequences of Primers Used in this Study

Designation	Nucleotide Sequence [a]	Enzyme
NS3	5'-GGGGAATTCGCC**ATG**GCGCCCATCACGGCCTACTC-3'	*Eco*RI
NS3-RI	5'-GTCGAATTC**CTA**GGTGACACATGTGTTACA-3'	*Eco*R1
NS3-R2	5'-GCACGAATTC**TCA**TGTGACGACCTCTAGGT-3'	*Eco*R1
53-S1	5'-GCCGCTCGAGGCA**ATG**GATGATTTGATGCTGTCCC-3'	*Xho*1
53-R1	5'GTGGGAATTC**TCA**GTCTGAGTCAGGCCCTTCTGT-3'	*Eco*R1
53-S2	5'-AGCCCTCGAGCCTTCCGGGTCACTGCC**ATG**GAGG-3'	*Xho*1
53-R2	5'-GAGCGAATTC**CTA**CCCTGGCTCCTTCCCAGCCTG-3'	*Eco*R1
53-R3	5'-CCAGGAATTC**CTA**CTTTGGCTGGGGAGAGGAGCTGGT-3'	*Eco*R1
53-R4	5'-TAGTGAATTC**TCA**GGGCAGCTCGTGGTGAGGCTC-3'	*Eco*R1

[a] The enzyme recognition sites are underlined. The translation initiation codons and complementary sequences of stop codons are shown in bold letters.

2.2. NS3-Expressing Stable Cell Lines

NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum and gentamicin (50 µg/mL) at 37°C in a CO_2 incubator. The cells were transfected with pH8-derived expression plasmids by calcium phosphate coprecipitation methods as described previously ***(28)*** and cultivated in the presence of a neomycin derivative (G418, 400 µg/mL, Gibco-BRL, Life Technologies Inc., Gaithersburg, MD) (*see* **Note 1**). As a control, NIH3T3 cells were transfected with pH8 vector plasmid. After 2–3 wk, stable transformant foci were cloned using cloning cylinders and cultured to bulk for further analysis. In some experiments, a mixture of transformant foci was obtained without subcloning. Expression of NS3 was confirmed as described below by using anti-NS3 monoclonal antibody (4A-3, kindly provided by Dr. T. Imagawa, Osaka University, Japan).

2.3. Detection of Apoptosis

1. Methanol.
2. 10 m*M* Hoechst 33342: Hoechst No.33342 (Sigma) dissolved in phosphate-buffered saline (PBS).
3. Lysis buffer: 10 m*M* Tris–HCl (pH 7.4), 10 m*M* EDTA, 0.5% Triton X-100.
4. 10 mg/mL RNase A (Nippon Gene, Tokyo, Japan).
5. 20 mg/mL proteinase K: proteinase K (Merck, Darmstadt, Germany) dissolved in lysis buffer.
6. 2% agarose gel.

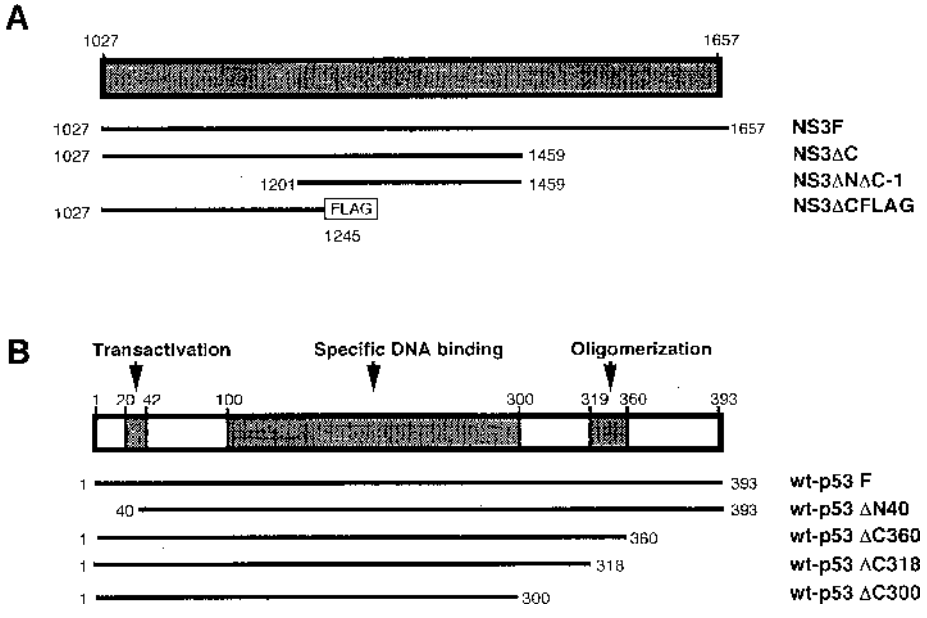

Fig. 1. Schematic representation of the open reading frame encoding the full-size and deletion mutants of NS3 and wt-p53: (A) NS3; (B) wt-p53. The numbers indicate amino acid positions. Various functional domains of wt-p53 are also shown.

2.4. Quantitative Apoptosis Assay

1. A 96-well plate (Becton Dickinson, Franklin, NJ).
2. 100 µg/mL actinomycin D stock solution: actinomycin D (Sigma Chemical Co., St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO) and stored at –20°C.
3. 5 mg/mL MTT stock solution: MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma) dissolved in PBS and stored at –20°C.
4. 0.04*N* HCl in isopropanol: 34 µL concentrated HCl in 10 mL of isopropanol.
5. Microplate reader: measure absorbance of samples at 570 nm.

2.5. Transient Expression in HeLa Cells by Vaccinia Virus–T7 Hybrid Expression System

1. HeLa cells.
2. Dulbecco's modified Eagle's medium (ICN Pharmaceuticals, Inc., Cost Mesa, CA).
3. Recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3) ***(29)***.

4. Lipofectin reagent (Gibco-BRL).

2.6. Indirect Immunofluorescence Analysis

1. Anti-NS3 monoclonal antibody (4A-3, kindly provided by Dr. T. Imagawa).
2. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (MBL, Nagoya, Japan).
3. A patient's serum that strongly reacts to NS3.
4. Anti-p53 monoclonal antibody (Ab-1 [clone 421]: Calbiochem, Oncogene Research Products, Cambridge, MA).
5. FITC-conjugated goat anti-human IgG (MBL).
6. Tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (MBL).

2.7. Immunoblot Analysis

1. Lysis buffer: 150 m*M* NaCl, 0.5% Triton X-100, 10 m*M* Tris–HCl (pH 7.5).
2. Polyvinylidene difluoride filter (PVDF) (Bio-Rad Laboratories, Hercules, CA).
3. 3% skim milk in PBS.
4. 0.5% Tween-20 in PBS.
5. Anti-NS3 monoclonal antibody (4A-3).
6. Anti-p53 monoclonal antibody (Ab-1 [clone 421]).
7. Peroxidase-labeled goat anti-mouse IgG (MBL).
8. Enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.8. Immunoprecipitation Analysis

1. 25 µCi of ^{35}S-translabel (Amersham).
2. RIPA buffer: 150 m*M* NaCl, 0.5% Triton X-100, 10 m*M* Tris–HCl (pH 7.5).
3. Anti-NS3 monoclonal antibody (4A-3).
4. Anti-p53 monoclonal antibody (Ab-1 [clone 421]).
5. Anti-FLAG monoclonal antibody (M2, Kodak).
6. Protein G/Protein A–sepharose (Calbiochem).
7. Lysis buffer: 50 m*M* Tris–HCl (pH 6.8), 100 m*M* dithiothreitol, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue and 10% glycerol.

2.9. Chloramphenical Acetyltransferase Assay

1. 0.05 mCi of ^{14}C-chloramphenicol (NEN Life Science Products, Inc., Boston, MA).
2. 4 m*M* acetyl coenzyme A: acetyl coenzyme A (Sigma) dissolved in 250 m*M* Tris–HCl (pH 7.8). Make fresh as required.
3. Ethyl acetate.
4. TLC plastic sheets (20 × 20 cm) silica gel 60 F_{254} (Merck) for thin-layer chromatography.
5. Chloroform (95%)/methanol (5%) solution.

3. Methods

3.1. Assay for the Suppression of Actinomycin D-Induced Apoptosis by NS3

Any cell line permissive for actinomycin D-induced apoptosis can be used. Prepare NS3-expressing cells and the control, as described in **Subheading 2.2.** Occurrence of apoptosis is determined by the following three criteria.

(i) Chromatin condensation and fragmentation of the nuclei

1. Cultivate NS3-expressing cells and the control at a concentration of 4×10^4 cells/well in an 8-chamber plastic slide overnight at 37°C in a CO_2 incubator.
2. Treat the cells with actinomycin D (30 ng/mL) and incubate for 48 h under the same condition as above.
3. Aspirate the medium and fix the cells with cold methanol at –20°C for 20 min.
4. Stain the cells with 1m*M* Hoechst 33342 and incubate at room temperature for 10 min.
5. Wash the cells three times with PBS and observe for morphological changes of the nuclei under a light microscope.

(ii) Fragmentation of Chromosomal DNA

1. Cultivate NS3-expressing cells and the control at a concentration of 1×10^6 cells/dish in a 100-mm tissue culture dish at 37°C in a CO_2 incubator.
2. Treat the cells with actinomycin D (30 ng/mL) and incubate for 48 h under the same condition as above.
3. Collect all adherent and floating cells from the dish.
4. Centrifuge the cells at 1500*g* for 10 min, discard supernatant by aspiration, and disrupt the cell pellet in 100 mL lysis buffer by pipetting several times and centrifuge at 11,000*g* for 20 min.
5. Transfer supernatant to an Eppendorf tube and treat it with RNase A (400 µg/mL) at 37°C for 1 h and then further treat with proteinase K (400 µg/mL) for another 1 h.
6. Add 20 µL 5*M* NaCl and 120 µL isopropanol to the treated supernatant and store at –20°C overnight.
7. Centrifuge at 11, 000 *g* for 15 min, discard supernatant, and dissolve the pellet in 20 µL of 10m*M* Tris × 1m*M* EDTA (TE) buffer.
8. Electrophorese the samples in 2% agarose gel containing ethidium bromide and visualize ladder patterns of DNA bands under ultraviolet illumination.

(iii) Quantitative Apoptosis Assay

1. Cultivate NS3-expressing cells and the control at a concentration of 1×10^4 cells/well of a 96-well plate at 37°C in a CO_2 incubator.
2. Treat the cells with actinomycin D (30 ng/mL) and incubate for 72 h under the same condition as above.
3. Add 10 µL of MTT stock solution to each well and incubate for 4 h at 37°C in a CO_2 incubator.

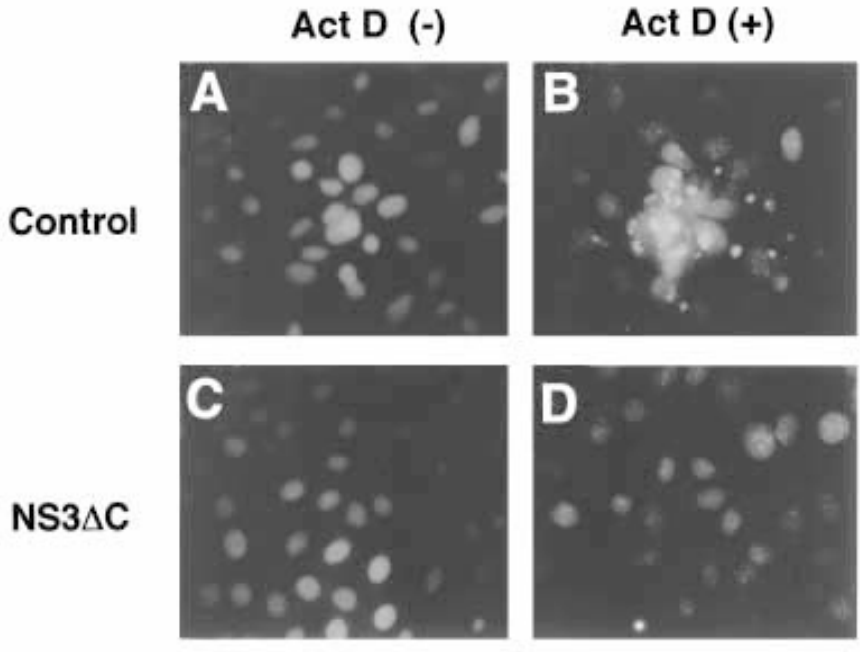

Fig. 2. Morphological changes of the nuclei after treatment with actinomycin D for 48 h: (A) untreated, nonexpressing control cells; (B) nonexpressing control cells treated with actinomycin D; (C) untreated NS3△C-expressing cells, and (D) NS3△C-expressing cells treated with actinomycin D. (Reproduced from **ref. *16***.)

4. Add 100 µL of HCl/isopropanol solution to each well, cover the plate with aluminum foil and incubate to dissolve the MTT formazans at room temperature overnight (*see* **Note 2**).
5. Measure the absorbance of each well at 570 nm using a microplate reader.

Representative results are shown as follows. Five independent NIH3T3 cell clones expressing NS3△C (CN3-1, -6, -10, -11, and -12) and five control clones (CpH8-12, -20, -25, -26 and -33) were examined. When treated with actinomycin D for 48 h, morphological changes of the nuclei were clearly observed in the control clones, but only slightly detected in the NS3△C-expressing clones **(Fig. 2)**. Consistent with the above result, DNA fragmentation was shown to occur more strongly in the control clones than in the NS3△C-expressing clones **(Fig. 3)**. Percent survival of the cells was monitored by quantitative apoptosis assay. No apoptotic cells were observed 24 h after actinomycin D treatment. After 48 h, apoptotic cells began to be detected in the control clones (20–50%), but only slightly in NS3△C-expressing clones (< 5%). The

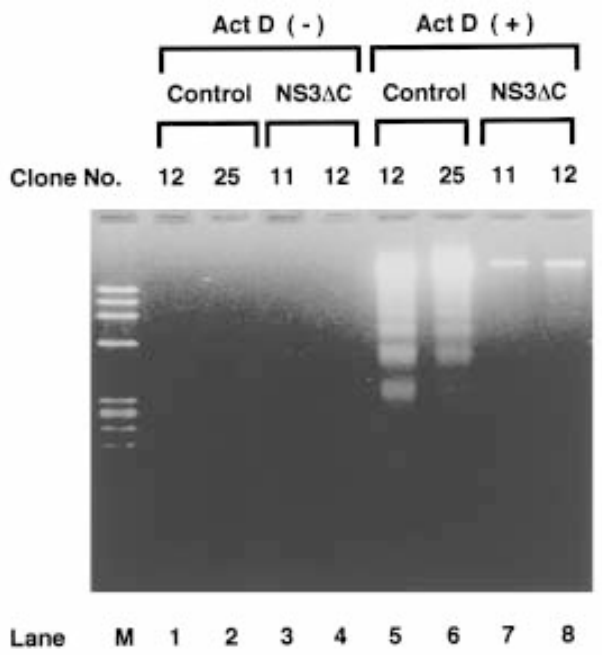

Fig. 3. DNA fragmentation after treatment with actinomycin D for 48 h. Lane M; size marker (ϕX174/*Hae*III digest); lanes 1 and 2; untreated, nonexpressing control cells, lanes 3 and 4: untreated NS3△C-expressing cells; lanes 5 and 6: nonexpressing control cells treated with actinomycin D; lanes 7 and 8: NS3△C-expressing cells treated with actinomycin D. (Reproduced from **ref. *16***.)

difference in the percent survival between the two groups became maximum after 72 h **(***see* **Note 3)**. The results obtained at 72 h are shown in **Fig. 4.** All of NS3△C-expressing clones were more resistant to apoptotic cell death than were the control clones. The difference in the average values of the percent survival between the two groups was statistically significant ($p < 0.01$).

3.2. Assay for the Suppression of Actinomycin D-Induced p53 Expression by NS3

1. Cultivate NS3-expressing cells and the control at a concentration of 4×10^5 cells/dish in a 60-mm tissue culture dish at 37°C in a CO_2 incubator.
2. Treat the cells with actinomycin D (30 ng/mL) and incubate under the same condition as above.
3. Wash the cells with PBS once and, using a cell scraper, collect adherent cells at different time points: 12, 18, 24, 28, and 32 h posttreatment.
4. Centrifuge at 11, 000 *g* at 4°C, discard supernatant and disrupt the cell pellet in

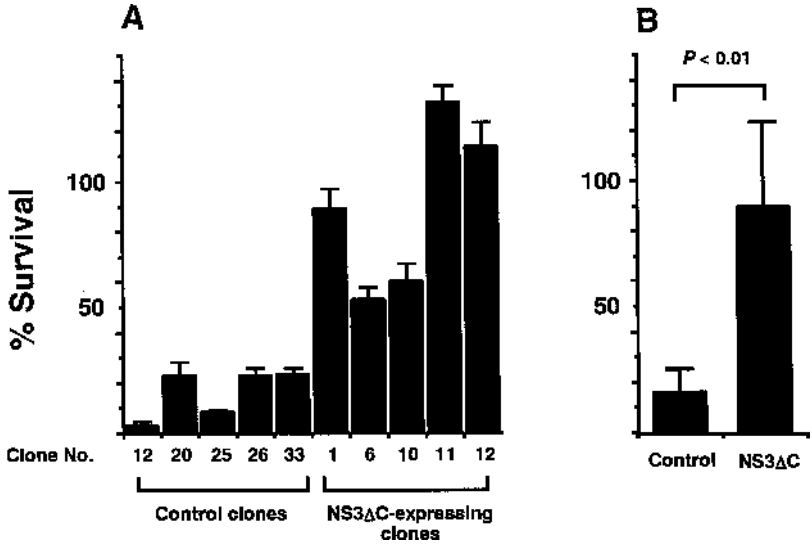

Fig. 4. Percent survival of cells after treatment with actinomycin D for 72 h: (A) percent survival of each clone; (B) mean values of percent survival and standard deviation for the five clones of each group. The difference between the NS3△C-positive group and the control was statistically significant ($p < 0.01$, χ^2 test with Yates' correction). (Reproduced from **ref. *16***.)

a lysis buffer by vortexing.

5. After clarifying by centrifugation, resolve the lysates by 10% SDS–polyacrylamide gel electrophoresis (PAGE).
6. Blot onto a PVDF filter electrophoretically at 4°C for 1 h.
7. Block the PVDF filter in PBS containing 3% skim milk overnight, and then wash with PBS containing 0.5% Tween-20 once for 10 min.
8. Incubate the filter with either anti-p53 or anti-p21WAF1 monoclonal antibody at room temperature for 1 h.
9. Wash the filter five times with PBS containing 0.5% Tween-0 for 10 min each, and then incubate with a peroxidase-labeled goat anti-mouse IgG at room temperature for another 1 h.
10. Wash the filter five times with PBS containing 0.5% Tween-20 for 10 min each and visualize the protein bands by a chemiluminescence method (ECL) according to the manufacturer's instruction.

An example follows. Although actinomycin D treatment for 28 h induced strong expression of p53 in the control clones, the same treatment induced significantly weaker p53 expression in all of the NS3△C-expressing clones **(Fig. 5A)**. The weaker induction of p53 expression in the NS3△C-expressing clones, compared with that of the controls, was first noticed after 12–18 h of actinomycin D

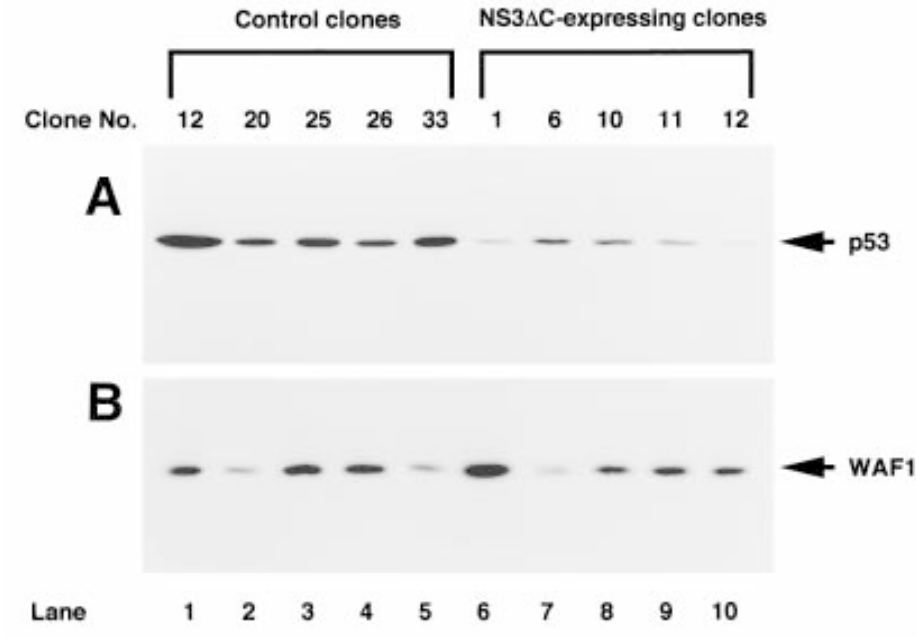

Fig. 5. Induction of p53 and p21WAF1 expression by actinomycin D treatment. p53 and p21WAF1 expression was determined by immunoblot analysis: (A) p53 expression after 28 h; (B) p21WAF1 expression after 28 h. Lanes 1–5: control clones; lanes 6–10: NS3△C-expressing clones. (Reproduced from **ref. *16***.)

treatment and became more apparent thereafter (data not shown). Expression of p21WAF1, a molecule known to be induced by p53, did not differ significantly between NS3△C-expressing clones and the controls, although certain degrees of clonal variation were observed in each of the two groups **(Fig. 5B)**. We assume that, in addition to p53, another mechanism(s) was regulating p21WAF1 expression.

3.3. Assay for the Colocalization of NS3 with wt-p53

Possible interaction between NS3 and wt-p53 can be examined by immunofluorescence analysis.

1. Cultivate HeLa cells to about 60–80% confluency at 37°C in a CO_2 incubator and infect the cells with a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3).
2. Prepare the following two solutions: solution A (dilute 2 µg of DNA in 100 µL serum-free medium) and solution B (dilute 20 µL of Lipofectin reagent in 100 µL serum-free medium). (*see* **Note 4**.)
3. Mix solutions A and B gently and incubate at room temperature for 30 min to form a Lipofectin–DNA complex. Add 800 µL of serum-free medium to the mixture.

4. Aspirate the medium 1 h postinfection, overlay the cells with the above mixture containing the Lipofectin–DNA complex, and incubate at 37°C in a CO_2 incubator for 12–16 h.
5. Wash the cells with PBS and fix with cold methanol at –20°C for 20 min.
6. Incubate the cells with anti-NS3 monoclonal antibody at room temperature for 1 h and wash three times with PBS.
7. Incubate the cells with FITC-conjugated rabbit anti-mouse IgG at room temperature for 1 h and wash three times with PBS. (For double-staining experiments, use anti-p53 mouse monoclonal antibody and patient's serum that strongly reacts to NS3 as first antibodies, and FITC-conjugated goat anti-human IgG and TRITC-conjugated goat anti-mouse IgG as second antibodies, respectively.)
8. Observe the cells under a fluorescent microscope.

Some examples follows: When expressed alone, NS3△C and NS3F were localized in both the cytoplasm and the nucleus, exhibiting granular and diffuse staining patterns in the cytoplasm and preferentially a granular staining pattern in the nucleus (**Fig. 6 A**,**D**). Interestingly, when coexpressed with wt-p53, NS3△C and NS3F were colocalized almost exclusively in the nucleus, as demonstrated by the double-staining immunofluorescence analysis (**Fig. 6B**,**C**,**E**,**F**). To examine whether or not an amino-terminal portion of NS3 was necessary for the nuclear accumulation of NS3, subcellular localization of NS3△N△C-1 was analyzed. In contrast to NS3△C and NS3F, NS3△N△C-1 showed only a diffuse staining pattern and was localized preferentially in the cytoplasm (**Fig. 6G**). Moreover, nuclear accumulation of NS3△N△C-1 was not observed even when coexpressed with wt-p53 (**Fig. 6H**), despite the nuclear accumulation of wt-p53 (**Fig. 6I**).

3.4. Assay for the Complex Formation Between NS3 and wt-p53

Complex formation between NS3 and p53 can be examined by immunoprecipitation analysis.

1. Coexpress each construct of NS3 and wt-p53 **(Fig. 1)** in HeLa cells using the method described in **Subheading 3.3., Steps 1–4**.
2. Twelve hours after transfection, label the cells with 25 µCi of ^{35}S-translabel/mL in serum-free medium for 3 h.
3. Aspirate medium, wash with PBS once, and collect the cells using a cell scraper.
4. Centrifuge the cells at 11, 000 *g* for 5 min at 4°C, discard supernatant, and lyse the cell pellet in RIPA buffer consisting of 150 m*M* NaCl, 0.5% Triton X-100, and 10 m*M* Tris–HCl (pH 7.5) (*see* **Note 5**).
5. Centrifuge the lysates at 11, 000 *g* at 4°C for 5 min. Collect the supernatant and incubate with either anti-NS3 or anti-p53 monoclonal antibody and 10 µL of Protein G/Protein A–Sepharose at 4°C for 1 h.
6. Centrifuge the mixture at 11, 000 *g* at 4°C for 1 min, remove supernatant, and wash six times with RIPA buffer.

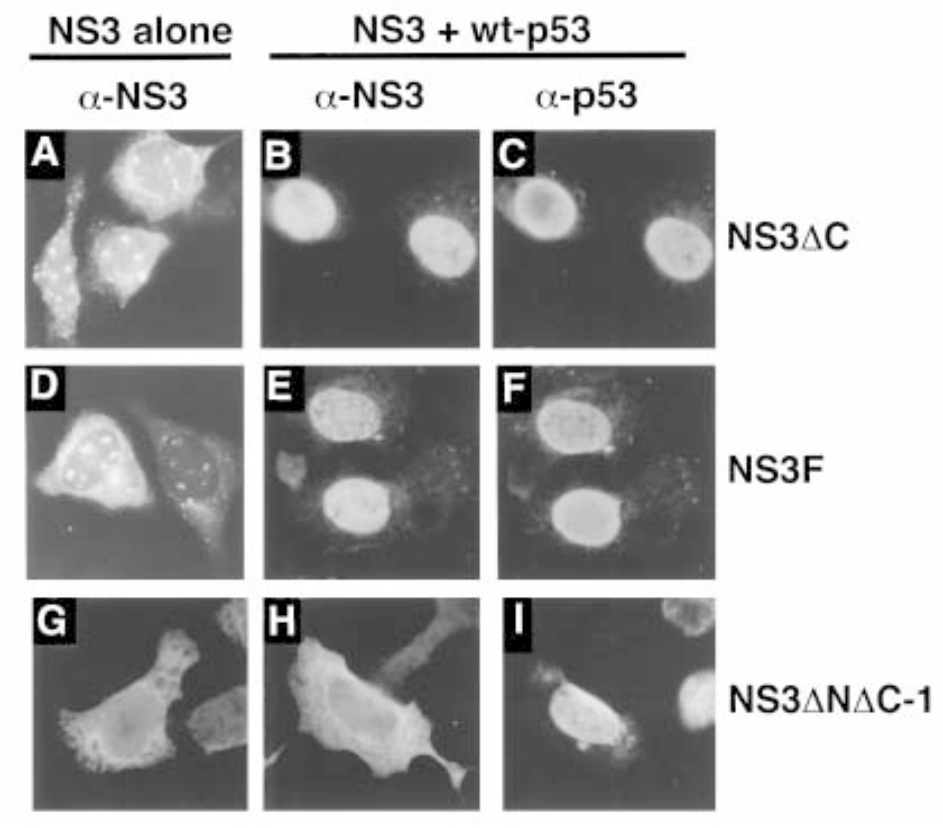

Fig. 6. Immunofluorescence analysis of NS3△C, NS3F, and NS3△N△C-1 in the presence or absence of wt-p53. (A,D,G) Cells transfected with pBSns3/1027–1459 (NS3△C), pBSns3/1027–1657 (NS3F), and pBSns3/1202–1459 (NS3△N△C-1), respectively, in the presence of pCDM8/neo (vector control) were analyzed using anti-NS3 monoclonal antibody. (B,C) Cells co-transfected with pBSns3/1027–1459 and pCDM8VAarg/neo (wt-p53), respectively, (E,F) cells cotransfected with pBSns3/1027–1657 and pCDM8VAarg/neo, respectively, (H,I) cells cotransfected with pBSns3/1202–1459 and pCDM8VAarg/neo, respectively, were each analyzed by a double-staining method. Panels B, E, and H: FITC staining with a patient's serum that strongly reacted to NS3; panels C, F, and I: TRITC staining with anti-p53 monoclonal antibody. (Modified from **ref. *17.***)

7. Add 10 µL of lysis buffer to the pellet and boil at 100°C for 5 min. Centrifuge at 11,000 *g* for 1 min and subject the supernatant to SDS-PAGE.
8. Dry the gel by a gel drier and visualize protein bands using the BAS 2000 system.

Some examples follow. Anti-NS3 monoclonal antibody coprecipitated wt-p53 with NS3F and NS3△C, but not with NS3△N△C-1 (**Fig. 7A–C**, lane 2). Likewise,

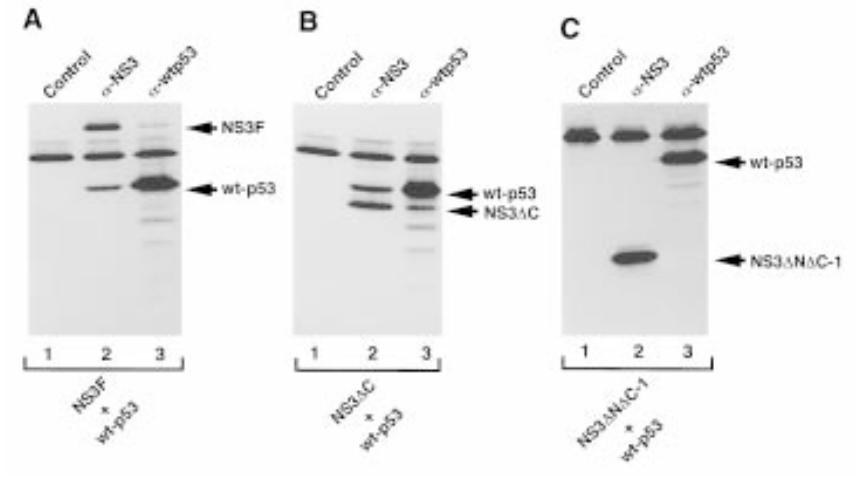

Fig.7. Complex formation between wt-p53 and various forms of NS3. Lysates of cells expressing wt-p53 and NS3F (A), NS3△C (B), or NS3△N△C-1 (C) were immunoprecipitated with normal mouse IgG (lane 1), anti-NS3 (lane 2), or anti-p53 monoclonal antibody (lane 3). Immunoprecipitates were separated by SDS-PAGE and analyzed by autoradiography. (Reproduced from **ref. *17***.)

anti-p53 monoclonal antibody coprecipitated NS3F and NS3△C, but not NS3△N△C-1, with wt-p53 (**Fig. 7A–C**, lane 3). Thus, NS3 was likely to form a complex with wt-p53 through an N-terminal portion of NS3 (amino acids 1027 to 1200). We then performed deletion mutational analysis of wt-p53. When coexpressed with NS3△C-FLAG, wt-p53△N40 was coprecipitated with anti-FLAG monoclonal antibody and, similarly, NS3△C-FLAG was coprecipitated with anti-p53 monoclonal antibody (**Fig. 8A**, lanes 2 and 3). When wt-p53△C300 was expressed with NS3△C-FLAG, however, coprecipitation of the two proteins was not observed (**Fig. 8A**, lanes 5 and 6). When wt-p53△C360 was expressed with NS3△C-FLAG, coprecipitation of the two proteins was clearly observed (**Fig. 8b**, lanes 1 and 2). However, when wt-p53△C318 was used instead of wt-p53△C360, coprecipitation of the two molecules became very faint (**Fig. 8B**, lanes 3 and 4). These results suggest that the C-terminal portion of wt-p53 (amino acids 301 to 393 or, more specifically, amino acids 319 to 360) is important for complex formation between NS3 and wt-p53.

3.5. Assay for the Inhibition of wt-p53-Mediated Transcriptional Activation by NS3

Inhibition of p53 function by NS3 can be examined by CAT assay. Used in this analysis is CAT-reporter gene under the regulation of bax promoter (pTM667-3), which has been known to be transactivated by wt-p53 *(27)*.

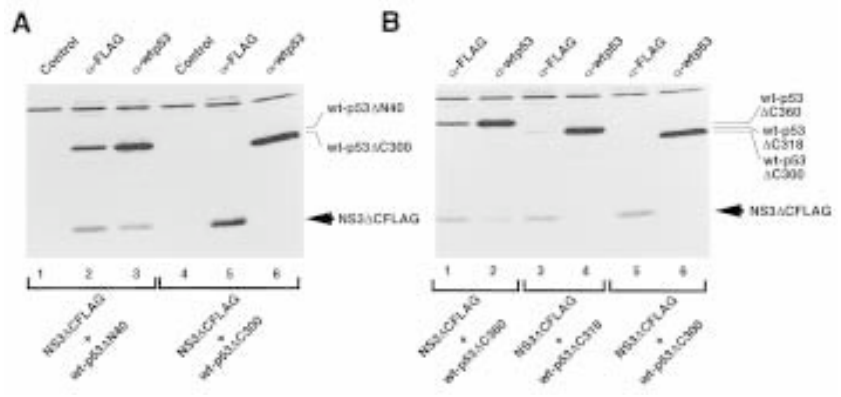

Fig. 8. Complex formation between NS3△C-FLAG and various forms of wt-p53. (A) Lysates of cells expressing NS3△C-FLAG and wt-p53△N40 (lanes 1–3) or wt-p53△C300 (lanes 4–6) were immunoprecipitated with normal mouse IgG (lanes 1 and 4), anti-FLAG (lanes 2 and 5), or anti-p53 monoclonal antibody (lanes 3 and 6). (B) Lysates of cells expressing NS3△C-FLAG and wt-p53△C360 (lanes 1 and 2), wt-p53△C318 (lanes 3 and 4) or wt-p53△C300 (lanes 5 and 6) were immunoprecipitated with anti-FLAG (lanes 1, 3, and 5) or anti-p53 monoclonal antibody (lanes 2, 4, and 6). (Reproduced from **ref. *19***.)

1. Cultivate NIH 3T3 cells at a concentration of 4×10^5 in a 100-mm-tissue culture dish at 37°C overnight in a CO_2 incubator.
2. Transfect the cells with expression plasmids for NS3 and wt-p53 together with the CAT plasmid (*see* **Note 6**).
3. After transfection, change the medium to fresh medium and incubate for 48 h under the same condition as above.
4. Aspirate the medium and collect the cells using a cell scraper.
5. Centrifuge the cells at 400 *g* for 5 min and discard the supernatant.
6. Resuspend the cell pellet in 80 µL of 250 m*M* Tris–HCl (pH 7.8), disrupt by sonication (160 W, 2 min), and centrifuge at 8000 *g* for 5 min to obtain cell extracts.
7. Mix 20 µL of each cell extract with 0.05 µCi of ^{14}C-chloramphenicol in the presence of 0.5 m*M* acetyl coenzyme A in 250 m*M* Tris–HCl (pH 7.8) and incubate at 37°C for 5 h.
8. Add 500 µL of ethyl acetate to the above mixture, vortex, and centrifuge at 8000 *g* for 5 min.
9. Transfer the upper phase to an Eppendorf tube, evaporate ethyl acetate in a vacuum evaporator for 1 h at 30°C, and dissolve the pellet in 30 µL of ethyl acetate.
10. Load the samples onto a silica gel for thin-layer chromatography.
11. Perform thin-layer chromatography in chloroform (95%)/methanol (5%) solution.
12. Visualize acetylated and nonacetylated forms of chloramphenicol by autoradiography and measure the radioactivity using the BAS 2000 system.

An example follows: After transfection with pTM667-3, CAT activity was not detected in cells not expressing wt-p53, but it was clearly detected in cells express-

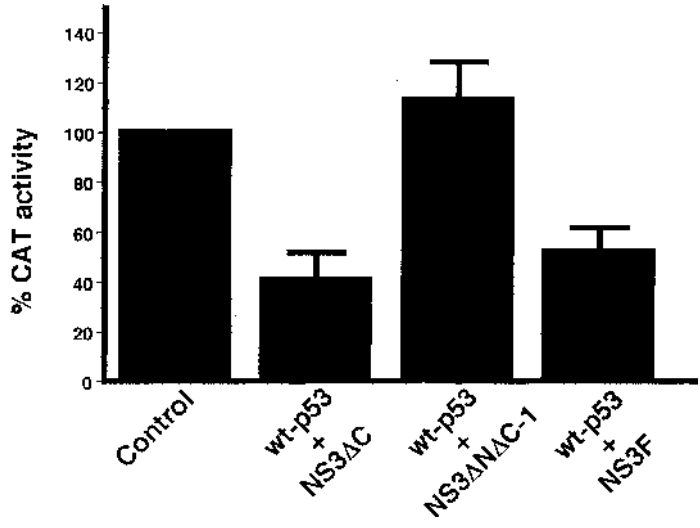

Fig. 9. Inhibition of wt-p53-mediated transcriptional activation by NS3. NIH3T3 cells were transfected with 0.5 μg of pTM667-3 (bax-CAT), 1 μg of pSG5p53 (wt-p53), and 15 μg each of test plasmids or pSG5 plasmid vector (control). CAT activity was measured as described in **Subheading 3.5.**, and relative activities are shown. Bars indicate the standard deviation.

ing wt-p53 (data not shown), with the results confirming the trans activation of the bax promoter by wt-p53. When NS3△C and NS3F were each coexpressed with wt-p53, CAT activity was decreased by 59% and 48%, respectively, as compared with the control in which wt-p53, but neither NS3△C nor NS3F was expressed **(Fig. 9)**. On the other hand, NS3△N△C-1, which did not bind to wt-p53 **(Fig. 7)**, did not influence the CAT activity. These results suggest that NS3 inhibits wt-p53-mediated transcriptional activation.

4. Notes

1. To obtain stable transformants expressing desired proteins, the concentration of a selection reagent is an important factor. The optimal concentration varies with different cell lines and, therefore, should be determined for each cell line. Cultivate cells in the presence of various concentrations of a selection reagent for 10–14 d. The minimum concentration at which all the cells have died is the optimal concentration for selection.
2. After forming crystals in viable cells, the MTT formazans are difficult to dissolve in the HCl/isopropanol solution. It will take 1 d to dissolve it completely after the dissolving solution is added.
3. When the MTT assay is used to determine the cell viability, you should keep in mind that this is an indirect method for measurement of cell viability. Under

some circumstances, the MTT assay may not actually reflect the cell viability. It is recommended to observe the cells under an inverted microscope before, the MTT assay is done.

4. In transient expression experiments using the vaccinia virus–T7 hybrid system, Lipofectin reagent is among the best to introduce plasmids into cultured cells. It is highly recommended that Lipofectin reagent be used in serum-free medium, because serum will decrease the transfection efficiency.
5. When you try to detect protein–protein interaction by using the immunoprecipitation method, one of the most important factors is the detergent used. If the protein–protein interaction is very weak, a strong detergent such as SDS will break it. In initial experiments, mild (2% digitonin) or moderate detergent (1% NP-40) is recommended to detect protein–protein association.
6. Transfection efficiency varies, depending on both quality and quantity of DNA. The use of highly purified DNA obtained by a fixed method is recommended if you want to compare biological effects of proteins expressed by different plasmids. Also, the total amount of DNA should be adjusted in each transfection by adding irrelevant DNA.

Acknowledgments

We are grateful to Dr. T. Imagawa (Osaka University) for providing monoclonal antibodies against NS3 and NS4A, Dr. H. Okayama and Dr. N. Hayashi (Osaka University) for providing HCV-BK146 and MKC1a, Dr. H. Saya (Kumamoto University) for providing pCDM8VAarg/neo, Dr. J. C. Reed (The Burnham Institute) for providing pTM667-3, and Dr. B. Moss (National Institute of Allergy and Infectious Diseases) for providing vTF7-3. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan, and a research grant from Research Foundation of Viral Hepatitis, Japan. This work was also part of the Large Scale Cooperative Study between Southeast Asian countries and Japan conducted by the Japan Society for the Promotion of Science.

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4

Abrogation of p53-Induced Apoptosis by the *Hepatitis B Virus X* Gene

Xin Wei Wang

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignant diseases worldwide and has become a leading cause for cancer-related deaths in adults from Asia and sub-Saharan Africa ***(1)***. The DNA tumor virus hepatitis B virus (HBV) has been implicated to play a major causative role in the development of HCC in man ***(2–4)***. The *HBx* gene, the smallest viral open-reading frame that may be essential for the viral life cycle ***(5,6)***, largely contributes the oncogenecity of HBV. The selective retention and expression of the *HBx* gene during acute and chronic hepatitis as well as in a great majority of HCCs may constitute an important step during HCC development ***(7,8)***. The oncogenic potential of the *HBx* gene has been experimentally demonstrated in a transgenic mice model ***(9)*** and in cell culture systems ***(10,11)***. *HBx* alone can induce HCC in certain transgenic mice ***(9)*** or can increase susceptibility to chemical carcinogens ***(12)*** and accelerate c-*myc*-induced HCC ***(13)***. Consequently, as an oncoprotein, *HBx* has been reported to disregulate cell-cycle transition ***(14,15)*** to potentially target certain proteases and proteasome ***(16–18)***, to interact with DNA repair factors ***(19,20)***, or to interact with the p53 tumor suppressor gene product ***(21–24)***.

The *HBx* gene, conserved among all mammalian hepadnaviruses, encodes 154 amino acids ***(25)***. One of the major cellular functions of *HBx* is its promiscuous transcriptional activation activity, a property that is believed to contribute to its oncogenecity. *HBx* does not bind to double-stranded DNA. However, a wide range of viral and cellular genes have been separately reported to be

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

upregulated by *HBx* ***(26–40)***. A "universal" effect of *HBx* on otherwise totally different types of promoter with no obvious consensus sequence has led to the hypotheses that *HBx* may regulate gene expression by interacting directly with host general transcription factors ***(20,41,42)***, or indirectly via activation of the cellular protein kinase C (PKC) and RAS–RAF mitogen-activated protein kinase signaling pathways ***(43–45)***. Although *HBx* can induce neoplastic transformation when overexpressed, it can also induce apoptosis in a p53-dependent or p53-independent manner ***(46,47)*** or sensitize cells to tumor necrosis factor α-induced apoptosis ***(48)***. Consistent with its role in apoptosis is the finding that *HBx* can suppress H–*ras*-mediated neoplastic transformation ***(47)***. These findings related to the pleiotropic effects of *HBx* are still highly debatable. Therefore, the precise mechanism(s) related to its effector remains largely obscure and many cellular activities mediated by *HBx* still remain controversial.

p53 is known to be important in safeguarding the genomic integrity of mammalian cells ***(49)*** by monitoring the G1 checkpoint ***(50)***, sensing DNA damage ***(51)***, assembling the DNA repair machinery ***(52)***, modulating gene amplification ***(53,54)***, or activating the cell-death program (apoptosis) to remove damaged cells ***(55,56)***. Apoptosis is a morphologically defined process that plays an important role in maintaining tissue homeostasis ***(57)***. The ability of p53 to induce apoptosis has been increasingly recognized as being important for its tumor-suppressor function ***(55,58–61)***. Loss of wild-type (wt) p53 function e.g., by mutation [reviewed in **refs.** ***62–64***], decreases cellular apoptosis induced by environmental and therapeutic agents that cause DNA damage ***(59,65–67)***. Inactivation of the p53 function through mutation or interaction with viral oncoproteins, therefore, is a common event during human carcinogenesis, which may provide a selective advantage for clonal expansion of preneoplastic and neoplastic cells.

Given the nature of p53 as a common target for various types of oncoviral proteins-mediated neoplastic transformation, including SV40 T antigen, E1A and E1B of adenovirus, and E6 and E7 of oncogenic HPV ***(68)***, it is reasonable to speculate that p53 may also be the target for *HBx*. Using an in vivo and in vitro model systems, we and others have previously shown that *HBx* can interact with p53 through the carboxyl terminal domains of p53 and *HBx* ***(21,22,69–71)***. To further establish the in vivo functional consequence following their physical interaction, we have developed a tissue culture experimental model system which primarily utilizes the microinjection technique to deliver various expression vectors into low-passaged nonimmortalized human cells. The powers of this technique are to avoid utilizing the immortalized cell lines that may generate ambiguity when assessing the cellular function of a particular gene and to easily introduce expression of a number of genes at a single cell level. By using this technique, we have confirmed previous findings that increased expression of

p53 predominantly induces apoptosis in primary normal human fibroblasts, renal epithelial cells, and hepatocytes, as well as Li–Fraumeni fibroblasts that have no endogenous p53 ***(70,72)***. The physiological significance of a p53-induced apoptosis in this system has been ascertained by the fact that many dominant-negative tumor-derived p53 mutants have lost this activity and also are able to block wt p53-induced apoptosis when coexpressed in the same cells ***(72)***. Therefore, when *HBx* and p53 are coexpressed in the same cells, the activity of a p53-mediated apoptosis is greatly reduced ***(70,73)***. Moreover, the *HBx* mutant that lacks the binding site for p53 has lost its ability to block a p53-mediated function ***(70,73)***. Therefore, this technique allows us to establish an in vivo significance for the interaction between *HBx* and p53. Our studies have been confirmed and extended by many other investigators ***(23,24,71,74,75)***. Our data indicate that inactivation of p53 by *HBx* may be an important step during liver carcinogenesis. Consistent with this model are the findings that a relatively infrequent mutation of p53 was found in hepatitis B virus positive HCC ***(76)***, and mutation of p53 was inversely correlated with the presence of *HBx* in HCC ***(77,78)***. In this chapter, I will mainly describe the use of the microinjection technique to study the functional interaction between p53 and *HBx*. Principally, this technique can also apply to examining genetically the function of any cellular genes. An example includes the use of this system to demonstrate the requirement of the XPB and XPD DNA helicases in a p53-mediated apoptosis pathway ***(72)***.

2. Materials

A successful microinjection depends on the use of a reliable and easy to use system. The specific model and types of equipment recommended here are based on my own experience and do not exclude other comparable items. The microscope should be chosen for visual clarity of the cells and comfortable positioning of the micromanipulator.

1. The Zeiss Axiovert 25 inverted microscope with 10 to 32X objectives is proficient for this purpose.
2. The semiautomatic Eppendorf micromanipulator (model 5171) combined with a microinjector (model 5242) **(Fig. 1B–D)** would be a good choice which is comfortably positioned onto the Axiovert 25 microscope. The microscope should be situated on a vibration-free table with an air isolation system.
3. The model PB-7 micropipet puller **(Fig. 1A)** and glass microcapillary tubes (model GD-1) from Narishige (Narishige, Sea Cliff, NY) are excellent for making the micropipets (*see* **Note 1**).
4. Earlier passaged primary human fibroblasts can be obtained from Coriell Cell Repositories (Camden, NJ). Primary human hepatocytes and renal epithelial cells can be obtained from Clonetics Corp (San Diego, CA). Primary human fibroblasts

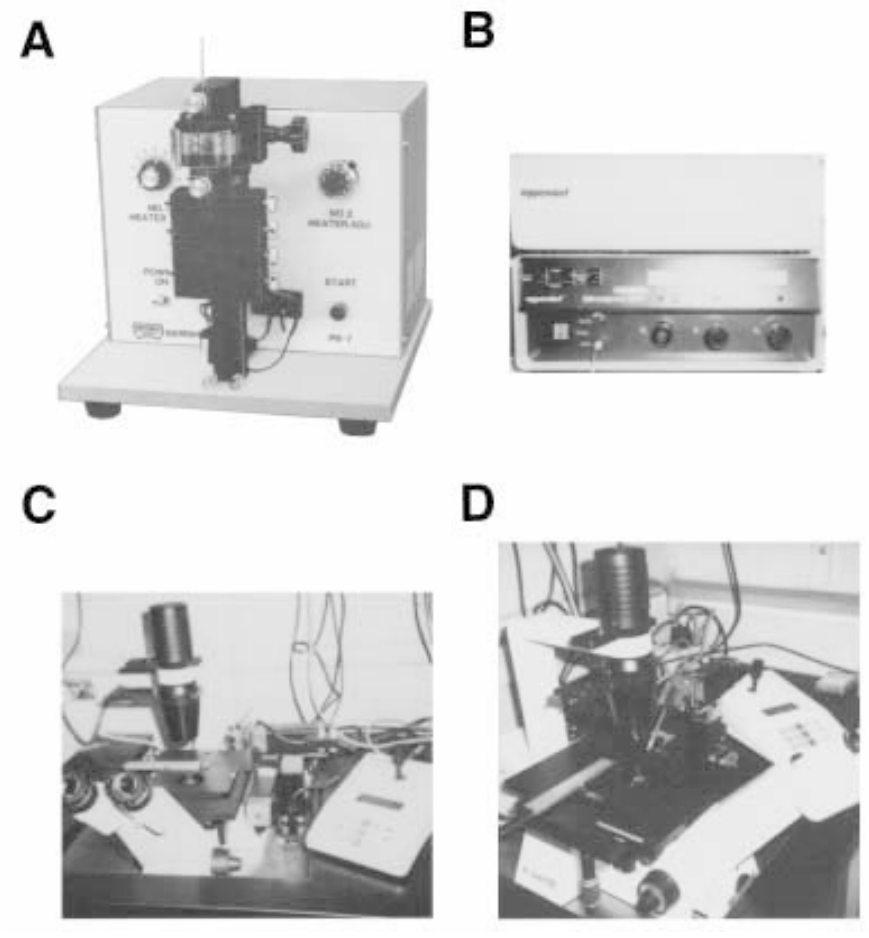

Fig. 1. A complete microinjection working station. (A) The Narishige needle puller model PB-7 for the preparation of the injected pipets is shown. (B) Microinjector 5242 by Eppendorf is equipped for manual or automatic operation with three pressure-control settings to adjust the injection, back, and clearing pressure. The front view (C) and the site view (D) of the Zeiss Axiovert 25 microscope, which has an Eppendorf micromanipulator mounted on the right of the microscope stage. An Eppendorf control panel with a joy-stick control is on the right side.

are grown in a monolayer in Ham's F10 medium supplemented with 10% fetal bovine serum (FBS), 2 m*M* L-glutamine, 100 units/mL penicillin, and 100 µg/mL of streptomycin. Primary human hepatocytes are grown in fibronectin-coated dishes containing serum-free HMM (Clonetics), and primary human renal epithelial cells are grown in serum-free REBM (Clonetics).

5. The HTC superCured printed glass microscope slides are available from CELLINE (Newfield, NJ). These slides should be cut into small pieces according to printed grids, cleaned by soaking with xylene for 5 min and methanol for 10 min, followed by air-drying, and sterilized by autoclaving.
6. Any mammalian expression vector used in this system should be purified by the double CsCl density gradient centrifugation protocol and suspended. The following plasmids have been used to study the functional interaction between p53 and *HBx*. Plasmid DNAs that contain a 1.8-kb human wild-type or mutant p53 cDNA under the control of cytomegalovirus (CMV) IE promoter were obtained from B. Vogelstein (Johns Hopkins University). Plasmid pCMV–*HBx* encodes a *HBx*-open reading frame from adr subtype of HBV ***(22)*** ***(see*** **Note 2)**.
7. Polyclonal antibody against p53 (CM-1) can be purchased from Signet Laboratories (Dedham, MA). Monoclonal antibodies against *HBx* were gifts of H. Will (Heinrich-Pette-Institut). Anti-rabbit or anti-mouse secondary antibodies conjugated with fluorescein isothiocyanate (FITC) or Texas Red can be purchased from Vector labs (Burlingame, CA).
8. Phosphate-buffered saline (PBS) plus buffer: 0.15 g glycine, 0.5 g bovine serum albumin (BSA) in 100 mL of PBS; stored at 4°C.
9. 16% paraformaldehyde stock (EM-grade) is available from Electron Microscopy Sciences (Ft. Washington, PA). Stock is diluted with PBS to 4% prior to usage.
10. The TUNEL assay: MEBstain apoptosis kit, available from MBL International Corp. (Watertown, MA), is used according to the manufacture's protocol.
11. FITC-conjugated Annexin-V can be obtained from BioWhittaker (Walkersville, MD).
12. Annexin-V assay binding buffer: 10 m*M* HEPES, pH 7.4, 140 m*M* NaCl, 2.5 m*M* CaCl2.

3. Method

Microinjection is a direct method for the delivery of any aqueous macromolecule into living cells. Basic procedures involve five steps which include (1) growing monolayer cultured cells on small glass cover slips, (2) preparing a capillary needle loaded with DNA, (3) injecting DNA into the nucleus of each cell with the microinjection equipment, (4) fixing cells following incubation and performing immunocytochemistry analysis with an appropriate antibody, and (5) analyzing injected cells to monitor the cellular phenotypes.

3.1. Cell Cultures

All cells are maintained at 37°C in a humidified atmosphere of 95% air : 5% CO_2. Fibroblasts and renal epithelial cells are routinely grown to 60–80% confluence, trypsinzied, and replated at 1 : 3 dilution. Primary hepatocytes are only used within the first passage. Trypsin-neutralizing solution (Clonetics) is employed when serum-free media are used. Cells are seeded onto the cover slip and incubated for an additional 2–3 d prior to microinjection. Logarithmically growing cells with no more than 70% confluence are essential for a successful microinjection.

3.2. Microinjection

1. Plasmid cDNA, in a concentration of 100–200 µg/mL suspended in PBS (pH 7.0), should be centrifuged for 10 min in Eppendoff microfuge prior to microinjection.
2. A micropipet is made fresh and an optimum micropipet can be generated from GD-1 glass microcapillary tubes by using a PB-7 micropipet puller with the settings of heater 1 at 30% and heater 2 at 70% and with all four blocks.
3. One microliter of plasmid solution is sufficient to be loaded onto each micropipet used for injecting into the nuclei of cells **(Fig. 2)**.
4. Microinjection is performed in open 60-mm culture dishes containing 12 mLof complete F10 media and a small cover slip containing cells. For the microinjector, the holding pressure should be 30–40 psi, the injection pressure 400 psi, and the injection time 0.5 s. By using these settings, it is estimated that each nucleus is injected with the DNA solution at a volume of about 10^{-15} L, which results in an average of 10–20 molecules per cell for the p53 or *HBx* expression vectors (*see* **Note 3**).
5. For each injection, routinely inject between 200 and 400 cells during a period of 30 min, which yields about 50–200 positive cells analyzed. The efficiency of microinjection ranges between 10% and 50%.
6. A longer period of injection is not recommended because of the alteration of pH value in the media. Many of the microinjections are carried out on the same slide when the wild-type is compared to various mutants or using the same needle when different types of cells are used to minimize variations resulting from differences in immunostaining, processing of the slides, vector copy numbers, and so forth.

3.3. Detection of the Positive Cells

1. Following microinjection, cells are incubated for a period of time (typically 24 h).
2. Cells are fixed at room temperature for 10 min in 4% formaldehyde (EM-grade, Electron Microscopy Sciences, Ft. Washington, PA), which is diluted in PBS, followed by incubation with methanol for 20 min (*see* **Note 4**).
3. The fixed cells can then be maintained in PBS at 4°C prior to the antibody staining.
4. These slides are incubated at 37°C for 1 h with PBS-plus buffer containing anti-p53 CM-1 antibody (1 : 200 dilution) and/or anti-*HBx* monoclonal antibodies (1 : 10 dilution).
5. Following five washes with PBS, incubate cells at room temperature for 30–60 min in dark conditions with the corresponding secondary antibodies, such as FITC-conjugated anti-mouse IgG (1 : 300 dilution) for *HBx* and Texas Red-conjugated anti-rabbit IgG (1 : 300 dilution) for p53.
6. Following five washes with PBS, add VectaSheild (Vector Labs) containing 0.5 µg/mL of 4',6-diamidino-2-phenylindole (DAPI) (Sigma), which counterstains the nuclei.
7. Analysis under the Zeiss Fluorescence microscope equipped with FITC, Texas Red, and DAPI filters.
8. The samples containing VectaSheild can be kept at –20°C under darkened conditions and the fluorescein intensity is stable for at least 6 mo.

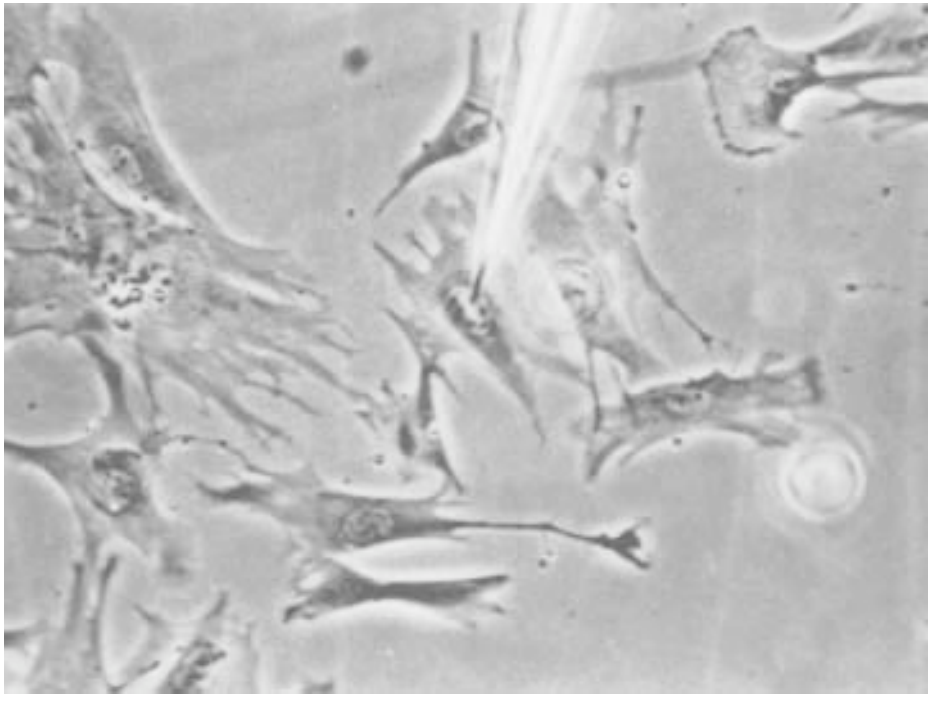

Fig. 2. Primary human fibroblasts with semiconfluence (viewed by a ×32 objective) are ready for microinjection. The pipet tip is smaller than the nucleolus of the cells and a change in the refractive index of the cell is viewed as it touches the membrane above the nucleus. The injection of the solution appears as a refractive change in the cell nucleus and no change should be observed in the surrounding cytoplasm. The pipet should leave the cells with no visible change after the microinjection is complete.

3.4. Analysis

1. p53-induced apoptosis is accessed by morphological alteration and biochemical markers. The biochemical markers include the terminal-deoxynucleotidyl-transferase (TdT)-mediated dUTP-biotin nick-end-labeling assay (TUNEL) and the Annexin V staining.
2. The typical characteristics of apoptosis associated with morphological alteration include cells with chromatin condensation, nuclear fragmentation, and apoptotic bodies **(Fig. 3)**.
3. Typically, among the p53 immunopositive cells analyzed at 24 h, an average of 20–30% of primary human fibroblasts, 40% of renal epithelium cells, and 20–50% of primary human hepatocytes display these characteristic features of apoptosis ***(70,73)***. At 72 h, more than 80% of cells undergo apoptosis.
4. The ability of a p53-mediated apoptosis can be efficiently abrogated by

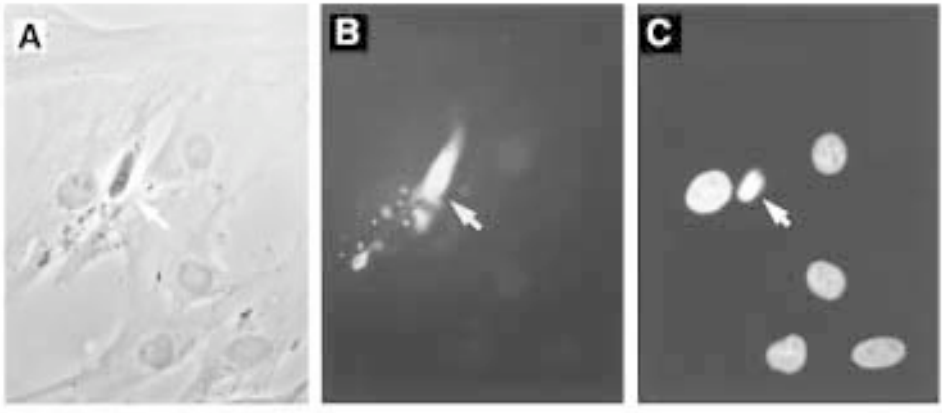

Fig. 3. Primary normal human fibroblasts undergo apoptosis following microinjection of the wt-p53 expression vector. Cells were injected with wt-p53 and were incubated for 24 h prior to fixation. p53 Protein was stained with CM-1 antibody (B). Nuclei were stained by DAPI (C). A phase-contrast image (A) of the same field (B,C) is also shown. Arrows indicate an apoptotic cell with a typical feature of apoptosis, including chromatin condensation, nuclear fragmentation, and apoptotic bodies. Photographs were obtained from an experiment performed by Elise Spillare (National Cancer Institute). Magnification: ×320.

coexpression with various dominant-negative p53 mutants (i.e., 143ala, 248trp, 249ser, and 273his) as well as by the wt *HBx* ***(70,72,73)***.

5. More than 95% of cells with morphological characteristic features of apoptosis are also positive for both TUNEL and Annexin V staining.
6. The TUNEL assay is performed using the MEBstain apoptosis kit.
7. FITC-Conjugated Annexin-V is used for Annexin V staining. Briefly, fresh cells are incubated for 5 min at room temperature under darkened conditions in the Annexin V binding buffer containing FITC-conjugated annexin V (1 : 100 dilution), rinsed once with binding buffer (10 min), and fixed for 10 min in the binding buffer containing 2% formaldehyde, 0.5% BSA, and 0.15% glycine, followed by a 5-min incubation with methanol. Cells are then stained with the appropriate primary and secondary antibodies as described earlier for identifying the injected cells.
8. For the negative controls, plasmids such as pactβgal (encodes a β-galactosidase gene) and pGreen-Lantern (encodes a green fluorescein protein, GFP) are used for microinjection. Anti-β–gal antibody (Life Technologies, Inc.) is used for visualizing the β-gal positive cells, whereas GFP- positive cells can be visualized directly under a fluorescence microscope using an FITC filter with or without fixation of the cells. The percentage of the apoptotic cells among the β-gal- or GFP- positive cells as well as various p53 mutants and *HBx*-positive cells are usually below 0.5% in our experimental conditions.

4. Notes

A pleasant and successful microinjection relies on the use of a good system, very healthy cells, nice micropipets, a very high quality of DNA, and uninterrupted time.

1. Although the ready-made micropipets from Eppendorf are handy, we found that it is much easier to efficiently and economically make our own reliable micropipets.
2. A high quality of plasmid preparation is essential for a successful microinjection. It is known that plasmids with broken ends are sufficient to induce endogenous p53 accumulation ***(79)***. Therefore, all the microinjected plasmids should be purified by double CsCl centrifugation protocol and suspend in sterile TE solution to avoid nicked DNA or to prevent the needle from clogging.
3. The ability of a gene-induced phenotypic alteration is usually not altered by the amount of the plasmids microinjected, which ranged from an average of 2 molecules per cell to 40 molecules per cell. However, an excess amount of DNA is also toxic to cells, resulting in a reduced microinjection efficiency. We routinely use a concentration of 100 ng/μL for the "inducer gene" (i.e., wt-p53) and a concentration of 100–200 ng/μL for the "anti-inducer gene" (i.e., mutant p53, *HBx*), and keep the maximum concentration of the injected DNA less than 300 ng/μL. A concentration of 300 ng/μL for all the negative control plasmids are used to avoid an "overdose" of the "effector genes." Under these conditions, expression should be detected following 3 h incubation.
4. Fixation protocol also is an important factor. The method used for p53 and *HBx* are optimized only for these proteins. When other genes are analyzed, different fixation protocols should be tested to set an optimum condition. Common protocols for an initial test include (1) methanol only, (2) acetone alone or combined with methanol, (3) paraformaldehyde only,(4) glutaraldehyde, and so forth. It is noticed that slides should never be kept dry after fixation prior to antibody staining. This step usually significantly reduces the background of the immunofluorescence staining.

Acknowledgments

I am grateful to E. Spillare, B. Vogelstein, and H. Will for their generosities in sharing the reagents and research materials. Special thanks also go to J. Coursen, L. Elmore, C. Harris, J. Hoeijmakers, W. Vermeulen, and J. Welsh for their inspiration, support, and advice on the research projects.

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5

Hepatocellular Carcinoma

Role of Hepatitis Viruses and Liver Cell Dysplasia

Carmen Vandelli and Francesco Renzo

1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver. HCC is a major problem worldwide because of its high prevalence and severe natural history and it is the major cause of cancer-related mortality in sub-Saharan Africa and in the Far East ***(1)***. HCC is the seventh most prevalent cancer in men and the ninth most common in women, with an estimated total of one million cases occurring each year ***(2–6)***. It carries a dismal overall prognosis: 92% of affected individuals will die because of their disease ***(3)***. In general, tumor incidence increases with age and occurs most often between the third and fifth decades of life ***(2)***. The distribution pattern shows striking and pronounced geographic and racial variations. The biological characteristics of this tumor vary significantly in different geographical areas, from country to country and even from one part of a country to another. Because of the widely divergent rates of HCC worldwide, it is possible to identify high-, intermediate-, and low-incidence areas. In China and south-east and sub-Saharan Africa, as many as 10–30 new cases per 100,000 males in habitants are reported each year, whereas in northern Europe, North America, and Australia, the annual rates of HCC are less than 3 cases a year per 100,000 males.

Several risk factors for HCC development have been identified, although the pathogenic mechanisms underlying carcinogenesis remain uncertain.

The marked geographic disparity in the incidence of HCC has suggested that environmental factors may play a part. In addition, individuals with a

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

Table 1
Factors Associated with an Increased Risk of Developing HCC

Age	Primary biliary cirrhosis
Hemochromatosis	Hepatitis virus infection
α_1–Antitrypsin deficiency	Exogenous hormone intake
Porphyria cutanea tarda	Ethanol ingestion
Glycogen storage diseases	Aflatoxin B1 ingestion
Cirrhosis	Wilson's disease
Dysplasia	Hepatotoxin exposure (thoratrast, carbon tetrachloride, polyvinylchloride)
Macroregenerative nodules	
Proliferation index (?)	

variety of hereditary biochemical abnormalities affecting the liver are known to be at increased risk of developing HCC *(7)*. The environmental hereditary factors related to HCC are listed in **Table 1**.

The major cause of the high incidence of HCC — apart from alcohol, possibly smoking, aflatoxins, oral contraceptives, and high doses of anabolic steroids, as well as genetic diseases such as hemochromatosis, α1-antitrypsin deficiency, tyrosinemia, or porphiria cutanea tarda — is viral hepatitis leading to cirrhosis and, eventually, to HCC.

Molecular, retrospective, and prospective epidemiological and clinical studies in human beings, hepadnavirus–infected animals, and transgenic mice models have confirmed the close connection between chronic hepatitis B virus (HBV) infection and the occurrence of HCC *(8–11)*. There is increasing evidence that HBV has both direct and indirect oncogenic effects and that, in all probability, the two commonly act in concert in the complex stepwise process of hepatocarcinogenesis *(12)*.

The sequential development of cirrhosis and HCC in patients with posttransfusion hepatitis *(13,14)* and the high prevalence of antibodies to hepatitis C virus (anti–HCV) in patients with HCC are clues pointing to HCV carriers as another significant population potentially at risk of developing HCC *(15–17)*. Although HCC may occasionally develop in normal liver *(11)*, most cases are associated with long-lasting chronic liver disease. The hepatocyte necrosis and mytosis of chronic hepatitis favor nodular regeneration that, in appropriate circumstances, is followed by hepatocyte dysplasia and HCC *(18,19, see also* **Note 1**).

Factors associated with chronic inflammatory and hepatic regenerative changes are important risk factors for hepatocarcinogenesis *(5,20–25, see also* **Notes 2** and **3***)*. Recent prospective studies *(26–29)* have also observed clinical

and biological predictive factors. In Western countries, HCC occurs in >80% of cases with underlying cirrhosis regardless of the etiology ***(1,30)***.

Many attempts have been made to detect possible precursors morphologically related to HCC. To describe some of these, the term liver cell dysplasia has been proposed by Anthony et al. ***(31)***, and it has been suggested that patients with liver cell dysplasia are at increased risk of developing HCC ***(32)***.

Because of the close association of liver cell dysplasia with HBV infection (see **Notes 2** and **4**, cirrhosis, and HCC, it has been suggested that liver cell dysplasia could be a preneoplastic lesion. A recent study has confirmed that the incidence of HCC is statistically more frequent in patients with liver cell dysplasia than in those without ***(33)***. In this chapter, the role of liver cell dysplasia and hepatitis viruses in developing HCC will be discussed and the methods used will be illustrated.

2. Materials

2.1. Clinical Specimens

Human liver tissues, collected from infected patients with HCV and/or HBV.

2.2. Reagents

1. Paraffin (Merck–Bracco, Milan, Italy).
2. Formaldehyde solution 40% (w/v) (Carlo Erba, Milan, Italy).
3. Alcohol.
4. Canada balsam mounting medium for microscopy (Sigma-Aldrich, Milan, Italy).
5. Distilled water.
6. Potassium iodide (Sigma–Aldrich, Milan, Italy).
7. Glycerin (Merck–Bracco, Milan, Italy).
8. Thymol (Merck–Bracco, Milan, Italy).
9. Acetic acid, glacial (Sigma–Aldrich, Milan, Italy).
10. Lipiodol-U.F. (Guerbet, Pharmades S.p.A., Italy).
11. Xylene (Merck–Bracco, Milan, Italy).
12. Eosin solution (Sigma–Aldrich, Milan, Italy).
13. Hematoxylin solution (Sigma–Aldrich, Milan, Italy).
14. VIDAS HBsAg reagent kit (bioMerieux Vitex, Missouri).
15. HCV 3.0 RIBA Kit (Ortho Diagnostic, Raritan, NJ).
16. α-Fetoprotein (Chiron Corporation, Emeryville, CA).

2.3. Reagent's Preparation

2.3.1. Hematoxylin Solution

1. Dissolve 25 g of potassium alum in 350 mL of distilled water with the aid of heat (Bunsen burner). When the salt has dissolved, withdraw the flame and add 0.5 g of Hematoxylin. Cool by plunging the container into cold water.
2. Dissolve 0.1 g of potassium iodide in 50 mL of distilled water.

3. Mix the two solutions together; add a crystal of thymol to prevent the growth of mold and add 100 mL of glycerin. Filter prior to use. The solution will ripen in about 10 d, after which it should be kept in a tightly stoppered bottle; the solution lasts for 2 – 3 mo.

2.3.2. Aqueous Eosin –Y Solution

Dissolve 1 g of Eosin –Y in 100 mL of distilled water and add 0.2 mL of glacial acetic acid. Filter before use. Solution is stable for 3–4 d. Use at room temperature.

2.4. Equipment

1. TSK Surecut modified Menghini aspiration biopsy set (17G × 90 mm) (TSK Laboratory, Ireland).
2. Flask.
3. Paper filter.
4. Slides.
5. Coplin jars.
6. Oven.
7. Microtome (E. Leitz, Wetzlar, Germany).
8. Speci-bath water bath (Folabo, Milano, Italy).
9. Light microscope.
10. Refrigerator.
11. Magnetic stirrer.
12. Centrifuge.
13. Sterile pipet.
14. Ultrasound scan (Acusan × P10, CT PACE, General Electric, Medical Systems).
15. Computed tomography (High Speed Scanner, General Electric, Medical Systems).
16. Magnetic resonance (RMN Signa 1.5T, General Electric, Medical Systems).

3. Methods

3.1. Diagnosis of HCC

Ultrasonography and a check of the serum α-fetoprotein level were performed every 6 mo in patients at highest risk to detect small hepatic tumors. When the serum α-fetoprotein level increased in a short time, and/or to above 250 ng/mL, or when a small or large hypoechoic nodules were detected by ultrasonography, further investigations were carried out by contrast computed tomography (CT), magnetic resonance (MR), and hepatic arteriography.

The sensitivity of enhanced CT scanning may be poor in early HCC because often the tumors are isodense with respect to surrounding liver tissue and are, therefore, poorly delineated.

Spiral CT scanning using intra-arterial contrast is useful in detecting hypervascular tumors and appears to increase sensitivity in tumors less than 1

cm. X-ray CT and magnetic resonance imaging are highly diagnostic for HCC, but they are generally less sensitive compared to angiography in recognizing a small cancer. Lipiodol angiography followed by CT appears to be one of the most sensitive modalities available to detect small HCCs. In this technique, a catheter is introduced into the hepatic artery, several milliliters of Lipiodol are slowly injected and CT scan of the liver is done after 10–14 d. Lipiodol is quickly removed from the non-neoplastic liver parenchyma, but cancer tissue is incapable of clearing it. As a result, Lipiodol remains in cancer tissue almost permanently producing a distinct contrast by X-ray, and a lesion as small as 2–3 mm may be detected by X-ray CT. A final diagnosis of hepatocellular carcinoma was based on biopsy specimens.

3.2. Liver Tissue Preparation

1. Liver biopsies obtained by ultrasound-guided or CT-guided percutaneous biopsy were fixed in 10% formalin for 24 h at room temperature.
2. Wash in running tap water for 1 h.
3. Dehydrate in graded alcohol (80% alcohol for 1 h; 95% alcohol for 1 h, absolute alcohol for 1 h).
4. Wash for 15–20 min in xylene.
5. Embed the tissue for 3–4 h in paraffin at 56–58°C.
6. When cool, cut histologic sections into 3- to 5- μm- thick sections on slides and stain with Hematoxylin & Eosin.

3.3. Hematoxylin & Eosin Staining Procedure

Before processing sections through the coloring agents, residual paraffin must be removed.

1. Xylene, three changes, 3 – 5 min in a Coplin jar.
2. Absolute alcohol, for 3 min.
3. 95% alcohol, 3 min.
4. 80% alcohol, 3 min.
5. Rinse in distilled water.
6. Stain in freshly filtered Hematoxylin for 10 min.
7. Rinse in distilled water and wash in running tap water for 10 min.
8. Rinse in distilled water.
9. Counterstain in Eeosin solution for 1 – 2 min.
10. Wash in distilled water for 30 s.
11. Dehydrate in three changes 80% alcohol, 95% and absolute alcohol, 2 min each.
12. Clear in xylene, two changes, 2 – 3 min each.
13. Mount in Canada balsam (the resin is dissolved 60% by weight in xylene) on the section.
14. Cover with cover slip.
15. Place the slide for 3 – 4 h in 37°C oven, before reading under a light microscope.

3.4. Liver Cell Dysplasia

The term *liver cell dysplasia* (LCD) was introduced by Anthony et al. ***(31)*** in 1973 to define a complex of hepatocyte morphological alterations of a putative malignant nature. Dysplasia was used to refer to atypical hepatocytes with cellular enlargement or to a combination of cellular enlargement, nuclear polymorphism with hyperchromasia and multinucleation of hepatocytes occurring in groups or whole cirrhotic nodules (**Figs. 1** and **2**). It was suggested that LCD might represent a precancerous condition and/or premalignant change ***(31,34–36)***.

Anthony et al. ***(31)*** found a close connection between LCD and HCC in an autopsy–surgical series from Uganda: LCD was found in 20% of the HCC-free patients with cirrhosis and in 65% of the patients with cirrhosis and HCC on resected specimens. Watanabe et al. ***(34)*** described clusters of atypical cells in chronic liver disease that were smaller than normal hepatocytes and had relatively large nuclei with a high nuclear/cytoplasm ratio. They called these cells "small dysplastic cells" and suggested that these are more likely to be candidates for the precancerous cells than are the cells of liver dysplasia, owing to their greater similarity to cancer cells.

In a recent study ***(33)***, the role of LCD as a potential precursor of HCC was prospectively evaluated in a number series of cirrhotic patients. Multivariate analysis showed that LCD was a major risk factor for the development of HCC, as cirrhotic patients with LCD were 3.83 times more likely to develop HCC than patients without LCD. The first line of evidence supporting the preneoplastic nature of LCD comes from experimental hepatic carcinogenesis in which preneoplastic lesions, such as the hyperplastic nodules or areas, are well-known entities that regularly precede tumor development in several models ***(37–42)***. Despite these findings, many investigators have concluded that there is no direct connection between liver cell dysplasia and HCC ***(43–45)*** and other studies have failed to confirm the progression from cirrhosis with LCD to HCC ***(45–48)***. Only small cell changes (SCC) have been considered a preneoplastic lesion ***(49)***.

In a recent study, Zhao and Zimmerman ***(50)*** demonstrated a phenotypic difference between large cell and small-cell liver dysplasia that supported the hypothesis that small-cell dysplasia is a precursor lesion in a hepatocarcinogenic pathway. However, this claim has not been confirmed by other studies ***(44,45)*** and needs further confirmation ***(49)***.

Recently, Ganne-Carrié et al. ***(51)*** confirmed by multivariate analysis that patients with cirrhosis and LCD were approximately five times more likely to develop HCC than patients without cirrhosis and LCD. The prevalence of LCD in this study was lower (13%) than that in the work of Borzio et al. ***(33)*** (24%) probably because of the small size of the liver specimens obtained and because of the limited number of HBV infections ***(51)***.

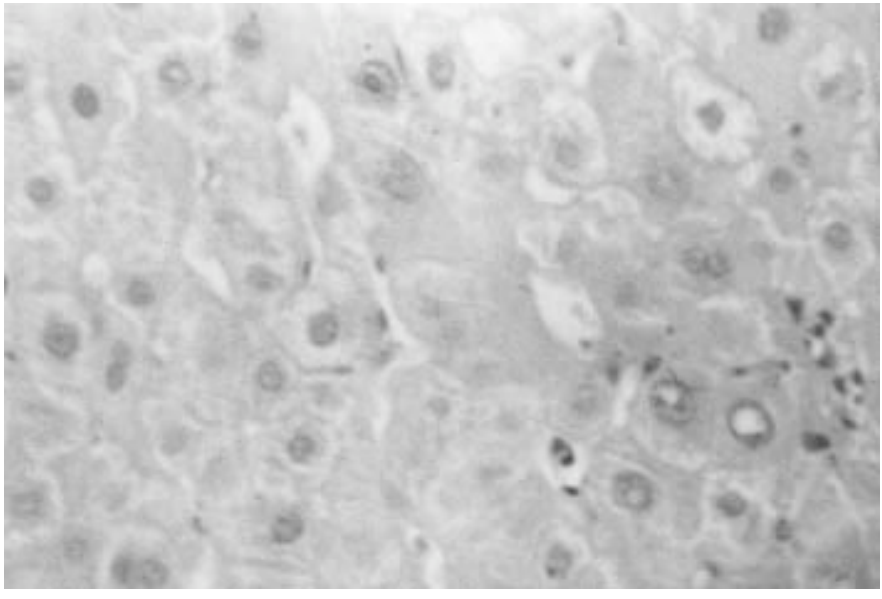

Fig. 1. Liver cell dysplasia characterized by a marked cellular pleomorphism, nuclear enlargement, and multinucleation (H & E; magnification, × 400).

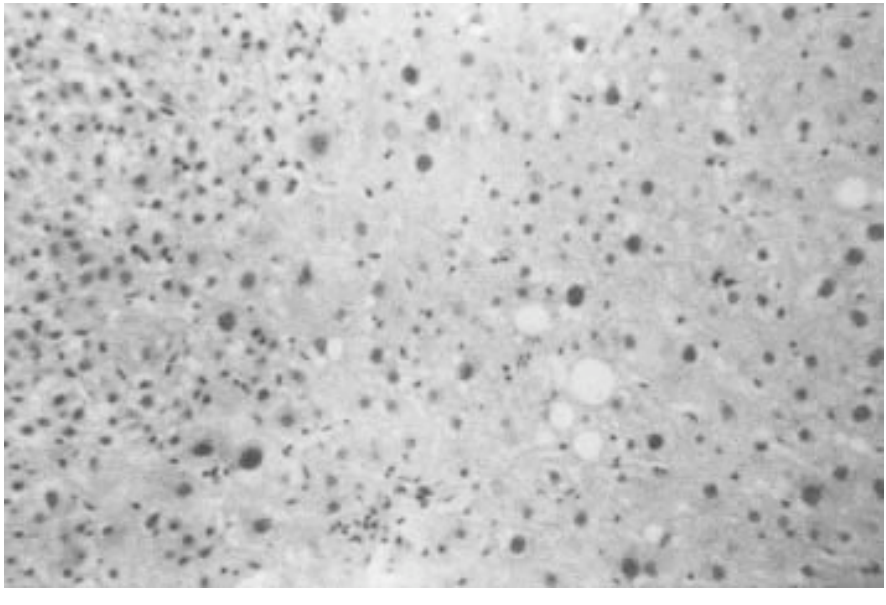

Fig. 2. Small liver cell dysplasia. Several small dysplastic cells can be observed showing hypercromasia and multinucleation (H & E; magnification, × 200).

So far, the association between LCD and HCC, first noted by Anthony et al. *(31)* has been confirmed by several cross-sectional studies *(43,48,52,53)* but denied by others *(45,46,54,55)*. Lee et al. *(56)* assessed the prognostic value of LCD for HCC through a matched-case control study that compared the occurrence of LCD between cirrhotic livers that were found either to harbor or to be free from HCC. The main conclusion was that LCD, independent of age, sex, and cause of cirrhosis, did indeed involve an increased risk of developing HCC with an estimated odds ratio of 3.3.

All longitudinal studies draw a similar conclusion: LCD involves a moderately increased (threefold to fivefold) risk of patient's developing HCC. This strongly suggests that LCD and HCC are linked in some underlying pathogenic fashion and argues against LCD representing merely a degenerative or regenerative alteration *(56)*.

In order (1) to assess the prevalence of liver cell dysplasia in patients with cirrhosis, (2) to evaluate the role of HBVand HCV infections in developing HCC, and (3) to determine whether the presence of LCD for liver cell change presaged any increased risk of HCC developing subsequently, we studied 113 patients (91 men and 22 women; mean age 38 ± 5.5 yr) with Child–Pugh class A cirrhosis. The diagnosis of cirrhosis was confirmed by biopsy in all patients and based on clinical criteria. The baseline characteristics of the patients are listed in **Table 2**. Liver cell dysplasia was found in 52/113 (46%) of patients with cirrhosis HBV and HCV related; 30.76% of patients with LCD and 3.27% of those without LCD developed HCC during follow-up **(Fig. 3)**. The characteristics of patients in relation to the development of HCC during follow-up are presented in **Table 3**. The mean follow-up time from diagnosis to HCC was shorter in patients with cirrhosis and LCD than in those with cirrhosis but without LCD. In our experience, LCD, a frequent feature of viral cirrhosis (both HBV and HCV related *see* **Notes 4** and **5**), was more frequently found in patients developing HCC *(57)*.

3.5. Assay for HBsAg Detection

Hepatitis B virus associated antigen (HBsAg) was detected by VIDAS HBsAg Kit (bioMérieux Vitex, Missouri) stored at 2–8°C, according to the manufacturer's instruction.

3.5.1. Assay Procedure

Store the VIDAS HBsAg kit at 2–8°C. Leave the unused components at 2–8°C.

1. Use the necessary number of SPRSs (solid- phase receptacle sensitized with monoclonal anti-HBsAg) and HBS strips from the refrigerator and allow them to

Table 2
Baseline Characteristics of the Patients

	No. of Cases	(%) Pecentage
Sex (M/F)	91/22	
Age (yr.; MD ± DS)[a]	38 ± 5.5	
Causes of cirrhosis		
HBsAg positive	36	31.8
HCVAb positive	75[b]	66.3
NANBNC	2	1.7
Child's class A	113	100
Liver histology		
Cirrhosis	113	100
Cirrhosis with LCD	52	46
Alcohol < 80grains/die	10	8.8
Iron overload	16	14.1

[a] MD ± DS = mean ± standard deviation.
[b] Four cases were HBsAg +/ HCVAb+.

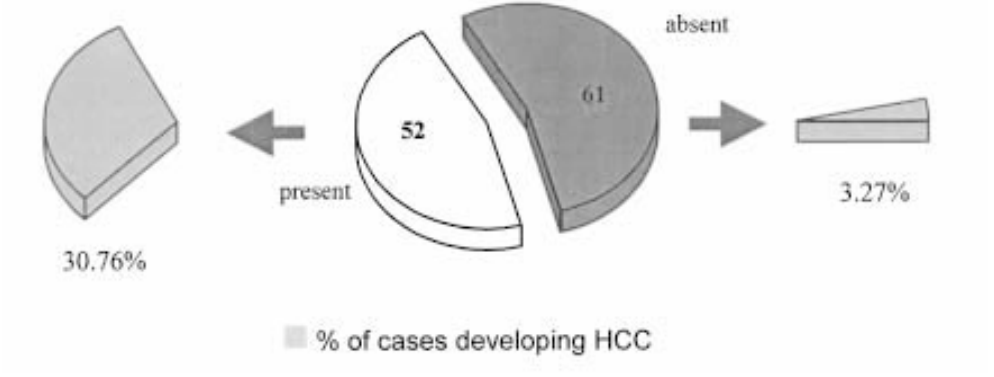

Fig. 3. Development of HCC in relation to the presence or absence of LCD. (From **ref. *57***.)

come to room temperature (at least 30 min).
2. Remove from the HBS– kit one HBS reagent strip and one HBS SPR for each sample, control, and standard to be tested. Make sure the moisture–proof SPR

Table 3
Characteristics of the Patients in Relation to the Development of HCC During Follow-up

	No. of Patients	
	With HCC (n = 18)	Without HCC (n = 95)
Sex (M/F)	15/3	76/19
HBsAg positive	5	31
HCVAb positive	13[a]	62[a]
NANBNC	0	2
Without LCD	2	59
With LCD	16	36

[a] Two cases were HBsAg+/HCVAb+.

pouch has been released after the SPRs are removed.
3. Place the HBS reagent strip and SPR in the VIDAS Transport Tray.
4. Enter the appropriate assay and patient's information on the VIDAS computer to create a work list. Type "HBS" to enter the assay code, and enter the number of the tests to be run. If the standard is being tested, type "S1" for the sample identification. The standard may be run in any position of the work list. The standard should be tested in duplicate if it is to be stored. If the positive control is to be tested, it will be scored by "C1." If the negative control is to be tested, it will be scored by "C2."
5. Vortex the standard, controls and samples. Before retesting sera, it is necessary to centrifuge them so as to eliminate interference caused by fibrin fragments or cell elements.
6. Mix strips thoroughly by inversing repeatedly. This manipulation must be performed after the strips have been left at room temperature for at least 30 min.
7. Pipet 150 µL of sample, standard or controls in to the sample well (note: samples and controls will be tested singly).
8. Slide the reagent strips into the channels that correspond to the VIDAS section positions indicated by the work list.
9. Initiate the assay processing as directed in the VIDAS operator's manual. The assay will be completed within approximately 65 min.
10. After the assay is completed, discard the used SPRSs and reagent strips into an appropriate biohazardous-waste container.
11. Once the assay is completed, results are analyzsed automatically by the computer.

3.6. Assay for HCV RIBA 3.0 Detection

The determination of the anti-HCV RIBA test was done by commercialy Kit Chiron RIBA HCV 3.0 SIA according to the manufacturer's instructions.

3.6.1. Assay Procedure

1. Approximately 30 min before beginning the assay, remove the kit from refrigeration (2 – 8°C) and allow the kit's components to come to room temperature (15 – 30°C).
2. Remove the required number of strips from the sealed foil pouches and place in the assay tube rack in their respective tubes. One tube per specimen and one tube each for the positive and negative kit controls are required. Kit-supplied positive and negative controls must be included each time specimens are assayed. Strips must be used within 2 wk after opening the pouch. Unused strips should be left in their original tubes, at 2 – 8°C, in a folded and resealed foil pouch with desiccant. Use tape to reseal the pouches, and record the date the pouches were opened and the expiration date of the strips on the pouch.
3. Prepare a record to identify the numbers on the strips with the corresponding specimen identification numbers.
4. Remove caps from tubes and add 1 mL of specimen diluent to each tube (make sure that the entire strip is covered with liquid).
5. Add 20 µL of the appropriate specimen or control to the corresponding labeled tube. Use a new pipet tip for each specimen or control aliquotted. Cap the tubes and invert to mix.
6. Place the rack with the tubes on a rocker and fasten with rubber bands or tape; rock (at 16–20 cycles/min) for 4 – 4.5 h at room temperature (15 – 30°C). Record the time started and the expected ending time of the specimen incubation step. The motion of the diluted specimen or controls over the strips, generated by a rocker, is important in achieving optimum performance of the assay. Periodically check to ensure that a rocking motion is maintained throughout the incubation. Improper functioning of the rocker, which may affect antibody binding, will invalidate the test results and require that the assay be repeated.
7. Uncap the tubes and completely aspirate liquid into a waste container. Add 1 mL of specimen diluent to each tube.
8. Cap the tubes and place the rack with the tubes on rocker and rock for 30 – 35 min at room temperature (15 – 30°C), then aspirate the liquid.
9. Add 1 mL of Working Wash Buffer to each tube, then pour liquid and strips into wash vessels containing 30 mL of Working Wash Buffer (maximum 20 strips per wash vessel).
10. Complete filling of the wash vessels with Working Wash Buffer (60 mL total volume), then decant the wash, making sure the strips are retained in the wash vessel. To retain strips, gently roll the wash vessel while decanting.
11. Add 60 mL of Working Wash Buffer, swirl, then decant the wash while retaining the strips as described in **step 10**.
12. Add 1 mL of conjugate per strip to each wash vessel (minimum 10 mL per wash vessel).
13. Rotate the wash vessels on a rotary shaker at 110 ± 5 rpm for 9 – 11 min at room temperature (15 – 30°C).
14. Prepare the working substrate per strip 1 h prior to use **(step 16)**.
15. Upon completion of conjugate incubation, decant the conjugate, then wash the

strips by adding 60 mL of Working Wash Buffer and swirling. Decant the wash and repeat this step two more times. Decant final wash.
16. Add 1 mL of working substrate per strip to each wash vessel (minimum 10 mL per wash vessel).
17. Rotate the wash vessels on a rotary shaker at 110 ± 5 rpm for 15 – 20 min at room temperature (15 – 30°C).
18. Decant the working substrate, then wash the strips by adding 60 mL of distilled or deionized water and swirling. Decant the wash and repeat this step one more time. To retain strips, gently roll the wash vessel while decanting.
19. Using forceps, transfer strips to absorbent paper and blot excess water. Let strips air-dry in the dark for least 30 min at room temperature. Interpret strips within 3 h.

3.6.2. Interpretation of Results

Anti-HCV reactivity in a specimen is determined by comparing the intensity of each antigen band to the intensity of the human IgG (levels I and II) internal control bands on each strip. The identity of the antibodies is defined by the specified location of the antigen band.

The intensity of the antigen/peptide bands scored in relation to the intensities of the internal IgG controls follows:

Intensity of Band	Score
Absent	–
Less than intensity of the level- I IgG control band	+/-
Equal to intensity of the level- I IgG control band	1+
Greater than intensity of the level- I IgG control band and less than intensity of the level- II IgG control band	2+
Equal to intensity of the level- II IgG control band	3+
Greater than intensity of the level- II IgG control band	4+

A negative, indeterminate, or positive interpretation is based on the reaction pattern present on the strip. For valid runs, the following criteria should be used for interpretation:

Antigen Band Pattern	Interpretation
No bands present having 1 + or greater reactivity or hSOD band alone having 1+ or greater reactivity Any single HCV band having 1+ or greater reactivity	Negative
hSOD band and one or more HCV bands having 1+ or greater reactivity	Indeterminate
A least two HCV bands having 1+ or greater reactivity	Positive

A band intensity less than the IgG control level I (i.e., +/–) is below the cutoff reactivity in the assay.

4. Notes

1. Many studies indicate that LCD influences the development of HCC, and hepatitis viruses contribute in some way to its development and progression. The close relationship between LCD and HCC suggests that LCD represents an alternative route for HCC development. Moreover, the finding that LCD coexists with an early acquisition of genomic alterations ***(82)*** supports its preneoplastic role. Shibata et al. ***(131)*** found HCC in 53% of patients with liver cells dysplasia as against 16% of patients without ($p = 0.003$), and by multivariate analysis, they found that irregular regeneration and diagnosis of cirrhosis were significant independent risk factors for HCC. The presence of LCD could predict HCC in patients with cirrhosis. Two prospective studies ***(39,42)*** demonstrated that in cirrhotic patients, LCD is an important risk factor in experimental carcinogenesis and precedes formation of hepatocellular carcinoma. However, other investigators have concluded that there is no direct connection between liver cell dysplasia and HCC, because LCD is too widely distributed to be regarded as a real precarcinomatous change. LCD remains a morphological finding of unknown pathogenesis ***(56)*** closely associated with HCC and viral hepatitis viruses.

   Further research in this area is essential to a better understanding of the role of viruses and liver cell dysplasia in human hepatocarcinogenesis and hence to enable the specificity and sensitivity of LCD for subsequent HCC to be established properly.
2. HBV and hepatocellular carcinoma. In advanced populations at low risk of developing liver cancer, HBV and HCV may account for 70 – 75% of liver cancer cases. Hepatitis B virus infection is considered to be the main precursor of HCC. Intensive epidemiological studies have supported a correlation between the high prevalence of chronic HBV infection and the high incidence of HCC in these areas, where there is a higher mortality due to HCC. In cohort studies, in which persons with chronic HBV infection are prospectively followed until HCC develops, the risk is between 7-and 110-fold greater ***(58,59)***. The majority of HCCs contain integrated HBV genome in the chromosomes, which points to the possibility of activation of cellular proto-oncogenes ***(60)***. Chronic hepatitis progressing to cirrhosis remains the most important precancerous etiological factor.

   Chronic infection with mammalian hepadnaviruses is associated with the development of HCC ***(61)***. Chronic carriers of HBV have a 200- to 300-fold greater risk of HCC than the general population ***(62)***. Molecular studies have demonstrated that HBV–DNA becomes integrated with host chromosomal DNA and can be found in the genome of tumor cells.

   There is controversy surrounding the putative direct role of HBV in liver cell transformation. Although no consistent pattern of integration has emerged and despite the fact that the HBV genome may integrate in different sites, it has been suggested that rearrangement of the host genome following HBV infection may play a role in liver cell transformation. Several HBV genes may play a role in

carcinogenesis. High levels of expression of the X protein are known to cause liver cancer in transgenic mice ***(63)*** with no background of hepatitis and cirrhosis. The X protein, under certain conditions, can transform hepatocytes and is able to activate the promoter of several cellular genes, such as c-*myc* and c-*jun*, which are related to cell growth. It has been suggested that the hepatitis B x antigen (HBx) may be a transactivator that increases the rate of transcription of oncogenes ***(64,65)***. Available data suggest that HBV proteins, such as the x proteins, either directly enhance mutagenesis or, alternatively, enhance the effect of environmental mutagens ***(66)***. Furthermore, integration of HBV–DNA can lead to chromosomal rearrangement of the site of integration ***(67,68),*** and human genes and proto-oncogenes are altered by HBV integration and may effect hepatocyte differentiation or cell-cycle progression ***(67–69)***. Tumor-suppressor genes, located on chromosome 17, such as the p53 oncogene, have been associated with HCC. The translocation of this chromosome is associated with HBV–DNA integration. Several studies have shown that HBV–DNA integration enhances chromosomal instability. Large inverted duplication insertions, translocations, and chromosomal deletions have been associated with HBV insertion ***(70–72)***.

This event results in a loss of important cellular genes (such as tumor-suppressor genes) ***(73,74)*** and other genes involved in regulating the growth of hepatocytes, an essential step for HBV-related carcinogenesis ***(71,75)***.

Several studies have suggested that overexpression of insulinlike growth factor-II (IGF-II) and its receptor may be an early event in HBV-associated hepatocarcinogenesis. Moreover, increased levels of transforming growth factor (TGF-α, $-\beta$) have been found in patients with HCC ***(76–78)***. Hsai et al. ***(77)*** have suggested that TGF-α expression is closely linked to HBV infection and that its enhanced expression is responsible for the process of hepatocyte regeneration and dysplasia involved in the carcinogenic process leading to an overt HCC ***(77,79,80)***. HBV induces the cancer through integration, transactivation, mutations in tumor-suppressor genes and increases in TGF-α ***(81)***.

Recently, Terris et al. ***(82)*** studied genetic alterations in large-cell and small-cell liver dysplasia and found that all cases of large-cell liver dysplasia displayed a polysomic population for chromosomes 1, 7, 17, and 18. The authors suggested that cellular modifications in large-cell liver dysplasia coexist with an early acquisition of genomic alterations, supporting the view that these phenotypic changes are preneoplastic.

3. HCV and Hepatocellular Carcinoma. The high prevalence of anti-HCV positivity in patients with HCC is another striking finding **(Table 4)** ***(83–89)***. Reports from Japan, Spain, and Italy have shown that 94.4%, 75%, and 65%, respectively of HCC patients were positive for anti-HCV ***(83,87,89)***. The frequent association of cirrhosis and HCC indicates that cirrhosis itself may play a role in the etiology or pathogenesis of HCC ***(90)***. However, there is growing evidence that cirrhosis is not absolutely necessary in the development of tumors and some studies indicate that HCC can arise in HCV-infected patients without histological evidence of cirrhosis ***(91–94)***.

Table 4
Prevalence of Anti-HCV Among Patients with Hepatocellular Carcinoma in Different Countries

Country	Anti-HCV+ %	Year	No. of Cases	Ref.
Italy	65	1989	132	83
	76	1989	200	84
	58	1991	78	85
	72	1991	88	86
Spain	75	1989	96	87
South Africa	29	1990	380	88
Japan	68	1991	109	89

Virally induced chronic necroinflammatory hepatic disease could contribute to neoplastic trasformation by increasing the hepatocyte turnover rate or because inflammation leads to local production of free radicals that may be mutagenic.

The hepatitic C virus may behave as a "promoting" factor by causing persistent liver cell necrosis and regeneration, resulting in cirrhosis and/or HCC ***(95)***.

The severity of liver disease correlates with the likelihood of developing HCC ***(28)***. Although cirrhosis is the main risk factor, macroregenerative nodules are particularly precancerous ***(54,96)***. Nodules with cellular or architectural atypia are indicative of malignant potential in cirrhotic livers ***(97)***.

One of the major debates surrounding hepatocarcinogenesis is whether HCV is directly carcinogenic or exerts its effects indirectly. The possibility that HCV may exert a direct oncogenic effect in the liver, leading to HCC in absence of cirrhosis, has been supported by the demonstration of HCV–RNA sequences in liver tumors that developed in normal livers ***(93)***. HCV is a plus-strand RNA virus and is not reverse-transcribed to DNA; hence, it is not integrated into the DNA host cell. The molecular mechanism of HCV-related transcription of hepatocytes remains obscure. HCV replication may mediate the coexpression of TGF-α and IGF-2 in cirrhotic livers and act as a possible initiating factor for hepatocarcinogenesis ***(98)***. Some of the proteins encoded by the HCV–RNA sequences may act like a proto-oncogene product. In vitro, it has been shown that expression of a region of the NS3 viral protein induces a transformed phenotype in NIH T3 cells ***(99,100)***. Furthermore, a recent study has demonstrated that the HCV capsid can cooperate with the c-Ha ras oncogene to transform rat embryo fibroblasts ***(100)***. The capsid of HCV might also interfere with the TNF pathway ***(101)*** and modulate the sensitivity of the cell to this cytokine.

The prevalence of HCV–RNA in the sera of patients with HCC in various countries is shown in **Table 5** ***(102–109)***.

Kiyosawa et al. ***(13)*** analyzed stored sera from patients who had developed acute posttransfusion NANB hepatitis and subsequent chronic liver disease and found that the average interval between acute infection and the diagnosis of

Table 5
HCV–RNA Prevalence in Serum of Patients with Hepatocellular Carcinoma

Author	HCV–RNA Positivity		Reference
	Anti-HCV (+) Cases (%)	Anti-HCV (–) Cases (%)	
Hagiwara et al.	27/29 (93)	5/10 (50)	102
Ruiz et al.	40/42 (96)	2/28 (7)	103
Sheu et al.	16/21 (76)	1/10 (10)	104
Takeda et al.	6/13 (46)	0/3 (0)	105
Thelu et al.	4/19 (21)	1/21 (5)	106
Benvegnù et al.	18/27 (67)	3/14 (21)	107
Bukh et al.	17/25 (68)	9/103 (9)	108
Mangia et al.	7/10 (70)	0/21 (0)	109

cirrhosis was 21.2 ± 9.6 yr and between acute infection and HCC, the interval was 29 ± 13.2 yr.

Tong et al. ***(14)*** in California followed 131 patients with chronic posttransfusion hepatitis C and found progression of the disease to HCC in 28.3 ± 11.5 yr after blood transfusion. Oka et al. ***(110)*** showed that 39% of 126 patients with cirrhosis developed HCC in 60 mo. In a retrospective study, we were able to demonstrate that HCV-correlated liver damage gradually worsens — 6 patients with steatosis developed chronic active hepatitis over a period of 4 to 9 years — whereas it takes on average about 24 yr from the time of infection for HCC to develop ***(111)***.

The annual rate of HCC occurrence in patients with cirrhosis has been shown to be more or less similar in Japan, Italy, and France, ranging from 3.0% to 6.5% per year ***(28,112–115)***.

Little is known about possible interactions between HBV and HCV in patients infected with both viruses with respect to the pathogenesis of HCC. Some studies indicate that the dual infection (HBV + HCV) results greater likelihood of HCC developing *(116-118)*.

4. Liver cell dysplasia and hepatitis B virus. A significant association between LCD and hepatitis B virus infection has been reported in a considerable number of studies. Anthony et al. ***(31)*** were the first to report this finding. Ho et al. ***(43)*** calculated an approximate HCC risk factor of about 13 : 1 if LCD was present; in males who were also HBsAg positive, the presence of LCD independently doubled the risk of HCC developing. In Japan, Akagi et al. ***(48)*** found that the prevalence of HBsAg was significantly greater in patients with LCD (70.2%) than in those without it (32.6%). Roncalli et al. ***(44)*** found a close connection between HBV infection and LCD, and with repeated biopsies in the some patients, they demonstrated the persistence of LCD. It worth noting that the relationship between LCD and

HCC is particularly close in areas with a high endemic diffusion of HBV infection.

Szczepansky ***(119)*** assessed the incidence of both types of liver cell dysplasia in autopsy material, including cases with cirrhosis and HCC. The occurrence of LCD was found to be significantly higher in cirrhosis with HCC than in cirrhosis without HCC, and a close relationship was seen between the presence of positive reaction for HBsAg and LCD.

Borzio et al. ***(33)*** conducted a prospective investigation into the role of LCD as an HCC risk factor. Their data indicate that cirrhotic patients with LCD are approximately four times more likely to develop HCC than patients without LCD. This study confirms the strict relationship between LCD and HBV, for the risk of neoplastic transformation was much higher in those patients with concomitant LCD and HBsAg. The appearance of LCD should therefore be regarded as a crucial event in HBV-related carcinogenesis.

5. Liver cell dysplasia and hepatitis C virus. Hepatocellular dysplasia has been implicated as a premalignant lesion in liver tissue, and it is a relatively common feature in the liver of individuals infected with HCV ***(54,120,121)***. The association between hepatitis C and LCD has been addressed in a few articles ***(122–124)***.

   The more rapid turnover rate of hepatocytes in cirrhosis increases the likelihood that a mutagen-induced DNA will occur. Evidence currently available suggests that HCV produces transformation indirectly by inducing chronic hepatic parenchymal disease ***(125)***. The process of recurrent hepatocyte necrosis and regeneration, with an increase in cell turnover, renders liver cells more susceptible to the adverse effect of other mutagenic agents ***(125)***. HCV may also exert a direct oncogenic action; indeed, HCV intermediates have been detected in HCC tumor and peritumoral tissues ***(126,127)***.

   The hepatitis C virus may induce genomic instability and favor mutations through its helicase activity ***(128)***. p53 Gene abnormalities have been reported in patients with HCV-associated HCC, which suggests that HCV may affect carcinogenic pathways via a p53 mechanism ***(129)***.

   Zhao et al. ***(50)*** found that in liver cell dysplasia, reactivity for the c-met protein was restricted to the small-cell type. In this study, E-cadherin staining was detectable in normal and large dysplastic hepatocytes, but not in small dysplastic liver cells, which supports the hypothesis that small-cell dysplasia is a precursor lesion in a hepatocarcinogenic pathway. Adachi et al. ***(130)*** evaluated cell-proliferative activity by immunostaining for proliferating cell nuclear antigen (PCNA) in HCC and demonstrated that increasing PCNA-labeling indices reflected an increasing histological grade of HCC; small-cell type liver dysplasia had PCNA-labeling indices similar to those of grade I and II hepatocellular carcinoma. Recently, Lee et al. ***(56)*** found that liver cell change (liver cell dysplasia) created a moderately increased risk of later HCC, and they proposed that liver cell change derives from derangements in a hepatocyte's normal process of polyploidization. Such derangements, possibly caused by chronic inflammation-induced DNA damage, could yield a population of enlarged liver cells with nuclear atypia and pleomorphism, frequent binuclearity, and minimal proliferation. Ac-

cording to this hypothesis, LCD would feature habitually in cirrhosis and regularly accompany HCC.

In our experience, there has been a strict correlation between LCD and HCC in HCV-positive cirrhotic patients: All 13 out of 18 cases who developed HCC were affected by anti HCV positive liver cirrhosis with dysplasia ***(57)***.

Acknowledgment

The authors wish to thank ASPImF (Associazione per lo Studio delle Patologie Infettive e metaboliche del Fegato) for financial support

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III

Molecular and Biological Characteristics

6

The Impact of Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV)

From Research to Clinical Practice

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1. Introduction

Hepatic carcinogenesis is a complex process that involves profound alterations of the hepatocyte genome (1,2) and has been graded in three stages: induction, promotion, and progression in the animal models (3–5). In recent years, the use of molecular biology techniques have improved significantly the understanding of the mechanisms (alteration of gene and gene expression) that occur during the different steps of carcinogenesis.

The hepatocyte transformation (induction), the first step of the neoplastic process, depends on genetic changes induced by viruses, chemicals, or increased cell turnover. As a consequence of genetic changes, the expression of proto-oncogens (ras, myc, fos) is altered and the cell cycle is deregulated (6–9). Such events, in spite of being irreversible at the single-cell level, can be controlled by apoptosis that removes altered cells from the liver (10). Also, the proliferation of initiated hepatocytes (promotion) that requires the continuous stimulus of promoting agents is still reversible (11). The promotion step can be inhibited by the interference of sex hormones, cytokines, enzymes like ornithine decarboxylase (ODC), or cyclins that are involved in hepatocyte proliferation (12,13).

The clonal expansion of foci of transformed hepatocytes represents the last step of progression to cancer. Growth factors (IGF-2, TGF-a, TGF-b) or their receptors may positively modulate the transformed hepatocyte proliferation

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

that is favored also by further genetic alterations of tumor suppressor genes, such as p53 (14–18).

1.1. Impact of Hepatitis Virus Infections on Hepatocarcinogenesis

Hepatitis viruses are thought to play a major role in hepatocarcinogenesis by both direct (induction) and indirect (promotion) mechanisms. Promotion sustained by necroinflammation and regeneration is active in any form of chronic inflammatory liver disease.

1.1.1. HBV and HCC Induction

Many in vitro and in vivo studies strongly support a direct role of HBV in all steps of oncogenesis, but particularly in the induction of HCC. The integration of HBV–DNA in the hepatocyte chromosomal DNA may alter hepatocellular DNA with several mechanisms. Random insertional mutagenesis with secondary chromosomal rearrangements or alterations of genes involved in proliferation and differentiation, and insertion into/near proto-oncogenes (as shown in the woodchuck model, in which HBV–DNA integrates always near the *myc* gene), can all be responsible for hepatocyte transformation ***(19–22)***. Furthermore, HBV could induce hepatocyte transformation transactivating cellular genes: several studies show that translation products of HBV–DNA (such as portions of S and X proteins) can, at least in experimental models, transactivate cellular genes and induce neoplastic transformation of cell lines ***(23,24)***. The fact that HBV–DNA integration may occur maintaining the integrity of these ORF (envelope region, enhancer I element, 5' sequence of the *x* gene), further supports the hypothesis.

1.1.2. HCV and HCC Induction

It remains to be clarified whether HCV also have a part in the induction of hepatocytes transformation or whether it is mainly implicated in the promotion and maintainance of hepatocyte proliferation.

In recent years a number of articles suggested that HCV is directly involved in the oncogentic process and that both structural and nonstructural proteins could be implicated. In in vitro experiments, the HCV core protein had been shown to transform fibroblasts interacting with the ras oncogene ***(25)***, to suppress gene expression, to modify the susceptibility of cultured cell to apoptotic signals ***(26–29)***, and, finally, to induce HCC in the transgenic mice ***(30)***. However, in these experimental models, the pattern and extent of expression of the HCV core protein are extremely different and higher then those usually observed during the course of HCV infection in humans. Also,

the NS3 protein was shown to be able to transform the NIH 3T3 cell ***(31)***, but at levels of expression significantly higher than those observed in natural HCV infection. Therefore, we think that the findings in favor of a direct carcinogenic role of HBV are numerous (obtained not only in vitro, but also in animal models and in humans) and consistent, like the evidence of unique clonal expansion of hepatocytes with integrated HBV–DNA during regeneration and HCC proliferation. On the contrary experimental data supporting a similar oncogenetic action of HCV is scarce and only generated in vitro. In conclusion at the moment, the most suitable oncogenetic effect for HCV appears an indirect one, being the virus responsible for maintainance of necroinflammation and regeneration, which promote the hepatocytes proliferation.

1.1.3. HBV, HCV, and HCC Promotion

We can measure the hepatocyte proliferative activity as a marker of HCC promotion by the quantitative analysis of silver-staining nucleolar organizer regions (AgNor) ***(32)***. This is one of several histochemical procedures that have been proposed and used to study hepatocyte proliferation. We previously showed that in patients with chronic virus-induced liver disease the presence of a hepatocyte mean AgNor area larger than 3 μm^2 (*see* **Note 4**) was significantly associated with the risk of developing HCC. Liver tumor was diagnosed, during a mean follow-up of 35.5 mo (range, 1–61 mo) in 56.2% of patients with a mean AgNor area larger than 3 μm^2 and in 11.8% of those with a mean AgNor area lower than 3 μm^2 ($p = 0.036$) ***(33)***.

To evaluate whether the infection with different hepatitis viruses is associated with different patterns of hepatocellular proliferative activity, we analyzed the results obtained by AgNor quantitation in 4 cohorts of 272 patients with chronic liver disease of different ethiologies (F. Oliveri et al., unpublished data). The prevalence of cirrhosis ranged between 22.9% and 100% and HCC developed during the follow-up in 0 – 33.3% of cases. In the cohort with a lower prevalence of cirrhosis and without HCC, it resulted that HBsAg-positive patients had a significantly higher proliferative activity as compared to anti-HCV positive or HBsAg/anti-HCV negative patients (mean AgNOR proliferation index 20.89 versus 6.09 and 6.5, respectively, $p = 0.0032$), independent of the presence of cirrhosis. Similarly, the proliferation index of HBsAg-positive patients was the highest when analyzed in the other three groups of patients, independent of the occurrence of HCC. Altogether, these findings suggest that HBV infection is associated with the highest AgNor values and independent of the presence of coinfection with HDV or HCV, cirrhosis, and the development of HCC.

The next step of the analysis was to study the impact of previous exposure to HBV in 71 HBsAg-negative patients: 26 patients with markers of exposure to

both HCV and HBV (anti-HCV, anti-HBs and/or anti-HBc positive), 17 anti-HCV positive, 13 anti-HBs and/or anti-HBc positive, and 15 negative for hepatitis B and C markers. The proliferation index was comparable within the different groups of patients when those without development of HCC or the whole cohort was considered. On the contrary, when patients who had HCC during the follow-up were considered, AgNor values were higher in individuals without HBV markers compared to those infected with HBV. The ratios of AgNor values of HCC patients to those of patients without HCC increased progressively for patients with markers of HBV past infection (HCC/noHCC ratio 1.12) to those without HBV markers with and without HCV infection (HCC/noHCC ratio 1.76).

These findings support the view that HBV plays a major role both in the induction and proliferation phases of hepatocarcinogenesis. In patients with ongoing or past HBV infections and with integrated HBV–DNA sequences that represent *per se* HCC risk factors, the development of HCC would require a lower promotional stimulus. On the contrary, in patients with exclusive HCV infection and without previous exposure to HBV, the development of HCC would need a higher proliferative stimulus. This could be sustained not only by necroinflammation and regeneration but also directly mediated by HCV, whose replication is associated with expression of TGF-α and ILGF II in the cirrhotic liver **(Fig. 1.)**. This hypothesis is in agreement with the observation (in patients who developed HCC) that the hepatocyte proliferation index was significantly higher in anti-HCV-positive and anti-HBc-negative patients than in anti-HCV/anti-HBc-positive patients.

Furthermore, the attack rate of HCC in anti-HCV cirrhotic patients is significantly reduced by the antiproliferative activity of interferon, only when the patients are missing any sign of previous exposure to HBV (anti-HBc negative). These results were obtained in a retrospective cohort study in which 451 anti-HCV-positive patients were considered ***(34)***: HCC developed in 5.2% of anti-HCV-positive/anti-HBc-negative patients treated with IFN as compared to 20.1% of those untreated; on the contrary, HCC occurred in 15% of anti-HCV-positive/anti-HBc-positive patients treated with IFN as compared to 17% of those untreated. The adjusted relative HCC risk in untreated versus treated patients was 6.28 for anti-HCV-positive/anti-HBc-negative patients and 1.53 in anti-HCVpos/anti-HBc pos patients. The mean size of HCC at the time of diagnosis was 2 cm in anti-HCV-positive/anti-HBc-negative patients as compared to 3 cm in the other patients. All these data support the hypothesis that a short course (median 7 mo) of IFN may affect the oncogenic mechanisms of HCV but not those of HBV. An early treatment and a longer duration of therapy could be most effective in HBV-infected patients.

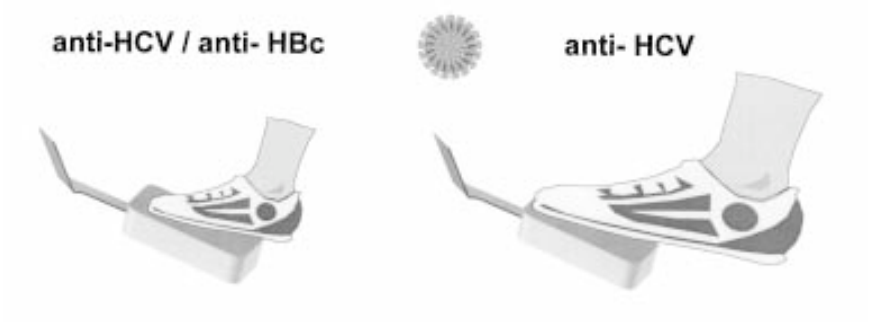

Fig. 1. Proliferative stimulus promoting HCC in anti-HCV positive patients with or without previous exposure to HBV. The induction of HCC associated with a past HBV infection determines a higher neoplastic risk, which explains the lower promotional stimulus observed in anti-HCV/anti-HBc positive patients. HCV could directly have a promotional effect, modulating the expression of growth factors.

1.2. Clinical and Diagnostic Implications

The characterization of some factors or mechanisms underlying the carcinogenetic process should not only improve the management of the HCC patient because of an early diagnosis of the disease, but it may also warrant more specific and efficacious preventive measure such as the identification of risk factors, the definition of tailored monitoring schedules, and preventive treatments.

We summarize in **Fig. 2.** some practical implications of the issues discussed. HBV appears to be involved in the induction of HCC via the integration of its DNA in the hepatocyte genome. Such an event is supposed to occur early during HBV infection; therefore, markers of both ongoing infection (as HBsAg/anti-HBc) and/or past exposure (as anti-HBs/anti-HBc or isolated anti-HBc) can be used in the clinical practice to identify individuals in whom the hepatocyte transformation process has been initiated. In anti-HCV-positive patients, the lack of evidence for HCV integration suggests the need for a continuous replication of HCV to warrant a HCC inductive effect. The demonstration of HCV–RNA (*see* **Note 3**) is therefore required to identify the HCV carrier with risk of cell transformation.

Sex hormones, cytokynes, and the altered expression of cyclins as factors implicated in the promotion of HCC can be identified as conditions at risk of development of HCC. In clinical practice, they are male sex, presence of liver necroinflammation (chronic hepatitis), and regeneration (cirrhosis). Furthermore, the persistence over time of serum markers of virus-induced liver disease (such as IgM anti-HBc and IgM anti-HCV [*see* **Notes 1** and **2**]), or the demonstration of abnormal iron metabolism and elevated alcohol intake would suggest the persistence of promotional stimuli in the patient.

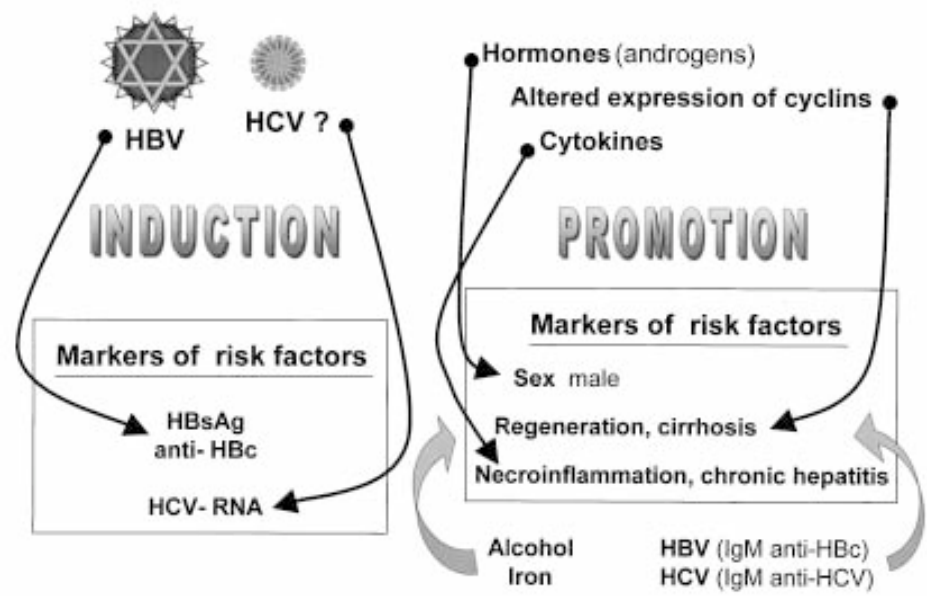

Fig. 2. Identification of risk factors for the development of HCC in clinical practice. Both HBV and HCV are supposed to be implicated in HCC induction; therefore, carriers of serum HBsAg, anti-HBc, or HCV-RNA can be considered to have a higher risk of HCC development. When signs of necroinflammation and markers of virus induced-disease (IgM anti-HBc or IgM anti-HCV) and hepatic regeneration (cirrhosis) are present, we are aware of the fact that the first two steps of carcinogenesis (induction and promotion) are active in the given patient. The simultaneous presence of signs of iron storage or elevated alcohol consumption would further increase the risk, because of the additive effect of these promoting agents.

In conclusion, in clinical practice the identification of the HCC risk resulting from HBV and HCV can be evaluated using simple and sometimes commercially available assays.

A deeper understanding of the mechanisms involved in the hepatocarcinogenesis warrants the possibility of a more incisive preventive and therapeutic intervention **(Fig. 3)**, even using simple and economic diagnostic tools.

Identification of liver disease carriers without previous exposure to HBV would warrant an active prophylaxis in these patients, virtually eliminating the possibility of having an additional inductive factor during the natural course of the disease. Characterization of the patients from the virologic point of view, identifying those with single or multiple virus infections, would suggest the

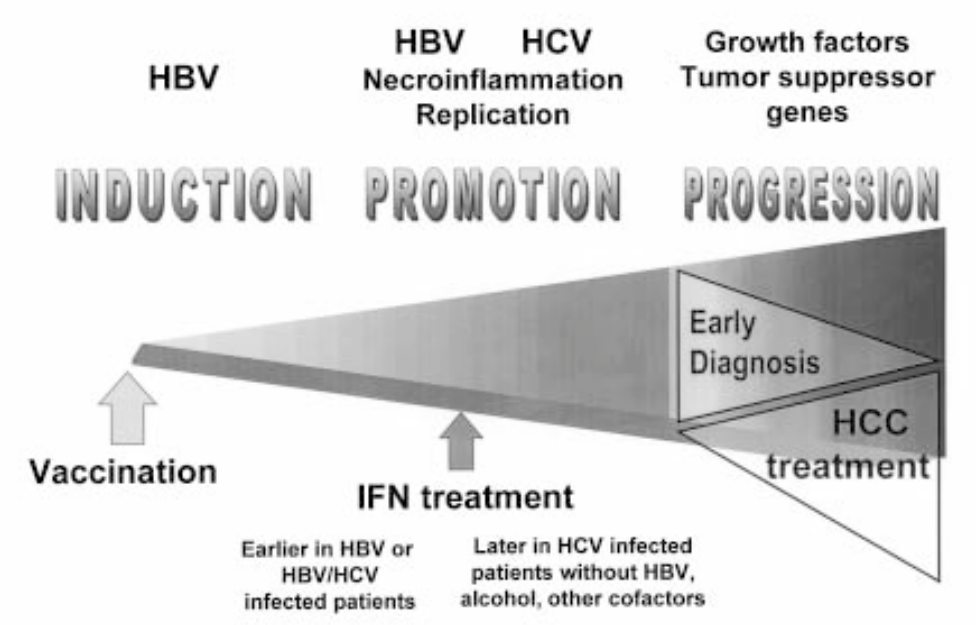

Fig. 3. Possible preventive or therapeutic interventions in different steps of hepatocarcinogenesis. Vaccination against HBV could eliminate one of the known factors able to induce hepatocyte transformation. IFN treatment inhibiting viral replication can reduce or eliminate virus-induced liver necroinflammation and inhibit hepatocyte proliferation. During the progression of HCC, an early diagnosis (favored by the improvement of the diagnostic procedure) would warrant an early and possibly more effective treatment.

more correct timing of IFN treatment. In anti-HCV-positive patients without other liver disease cofactors (and anti-HBc-negative), treatment could be delayed in more advanced phases of disease and both the antiviral and antiproliferative activity of interferon will be effective. On the contrary, in patients with HBV infection or with both HBV and HCV infections, the treatment should be started as early as possible, trying to block or at least reduce the progression of liver disease.

Not only primary but also secondary prevention can be optimized with an early diagnosis and possibly a more effective treatment. In addition in chronic viral hepatitis, it is possible to define the individual risk of HCC and to tailor the monitoring schedule. To do it we have to take into account the role of the different factors implicated in hepatocarcinogenesis **(Fig. 4.)**: number of viruses, other cofactors, duration of the induction phase, duration of cirrhosis, and necroinflammation ***(35)***.

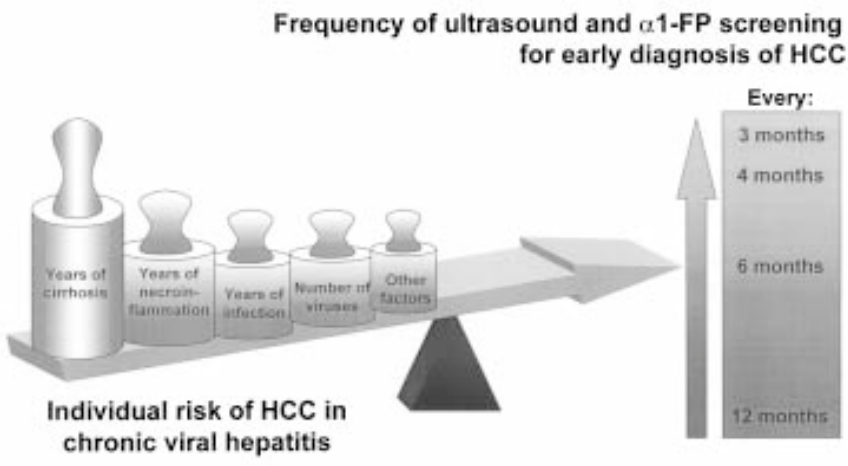

Fig. 4. Identification of the individual risk of HCC in the patient with chronic viral hepatitis to tailor the monitoring schedule. An equation has also been defined with this purpose: $y = K_1x+n(K_2-K_1)(x-a)+K_3(b+c)(1+n)+z$, where y = risk index, x = age (yr), a = age of onset of hepatitis virus infection (yr), b = duration of cirrhosis (age), K_1 = overall incidence of HCC in individuals of the area, K_2 = incidence of HCC in patients with chronic hepatitis virus infection, but without cirrhosis, K_3 = incidence of HCC in patients with cirrhosis and chronic hepatitis virus infection, n = number of hepatitis viruses, and, z = other known (as proliferative activity as measured by AgNOR) and unknown factors influencing the development of HCC.

2. Materials

Serum specimens obtained during follow-up from patients with chronic liver disease, mainly of viral ethiology

Liver biopsies obtained from patients with chronic liver disease, mainly of viral ethiology

3. Methods

3.1. HBsAg and Anti-HBc Detection

Immunometric, radiolabeled or enzymatic standardized assays are commercially available. They have a high degree of sensitivity and specificity as required by screening assays for blood banks.

3.2. IgM Anti-HBc Dectection

Commercially available assays had been designed to detect high levels of IgM antibodies as found during acute hepatitis or severe hepatitis exacerbations. The specificity of most of the assays is high, being based on the specific IgM capture by an anti-μ, followed by incubation with HBcAg and recognition by anti-HBc antibodies (sandwich method).

3.3. IgM Anti-HCV Detection

The assay had been already defined to detect the antibodies in patients with chronic disease. The antigen is directly coated on the solid phase and reactive sera are identified by the anti-μ antibodies ***(36)***.

3.4. HBV–DNA Detection

Three techniques can be used: probe hybridization (sensitivity limit 10^6 genomes/mL) ***(37)***, signal amplification (sensitivity limit 10^5 genomes/mL), and nucleic acid amplification assays (sensitivity limit ranging from 10^3 – 10^2 genomes/mL according to the protocol) ***(38)***. For the first and the third techniques, both in-house and commercial assays are available.

In clinical practice HBV–DNA can be detected and quantitated in most of the patients with the two less sensitive techniques; however, in HBsAg/anti-HBe-positive patients, viremia detected by these assays can be temporarily undetectable. A correct clinical use of nucleic acids amplification requires a quantitative detection or the definition of the assay sensitivity: in fact, low levels of HBV–DNA ($<10^3$ gen/mL) can be detected also in healthy carriers of HBV, whereas levels higher than 10^3 gen/mL are found in carriers with disease ***(39)***.

3.5. HCV-RNA Detection

Two techniques can be used: signal amplification (sensitivity limit 10^5 genomes/mL) and nucleic acid amplification assays (sensitivity limit ranging from 10^3 to10^2 genomes/mL according to the protocol) ***(40–46)***. Commercial assays are available for both the techniques; several in-house protocols have also been defined for nucleic acid amplification ***(47)***. In the last 2 yr efforts ***(48,49)*** have been made to reach an acceptable degree of standardization, but at the moment, it is lacking a common standard that would be necessary to warrant a correct quantitation and to have a comparable definition of the assays sensitivity.

3.6. Hepatocyte Proliferative Activity

Quantitative analysis of silver-staining nucleolar organiser regions (AgNOR) can be performed by measuring the interphase AgNOR numbers on routine histological sections stained by the silver method according to Ploton ***(33)***. The specimens are fixed in buffered formalin. Silver staining is carried out using a solution of 1 volume 2% gelatine in 1% aqueous formic acid and 2 volumes of 50% silver nitrate. The staining reaction is performed for 14 min at 37°C. Thereafter, the area occupied by the interphase AgNOR within nuclei of 100 cells is measured using a specific program (IM 5200) of a computer-assisted image-analysis system ***(33)***.

4. Notes

1. IgM anti-HBc detection. Most of these assays have a high analytical sensitivity, but the clinically diagnostic cutoff has been artificially elevated to identify only serum samples with high levels of antibodies. Therefore, to detect the low levels of antibodies usually present in patients with chronic hepatitis, it is mandatory to use not only assays with an adequate analytical sensitivity but also with a cutoff and a grey zone defined for diagnosis of chronic hepatitis B (IgM anti-HBc levels ranging from 100 to 7 Paul Erlich Institute Units) ***(50,51)***. Otherwise, the risk of missing the identification of patients with ongoing HBV-induced liver disease is significant and it cannot be covered by detection of serum HBV-DNA, which is a marker of HBV replication only. In fact, using assays with low sensitivity (hybridization technique), patients with low levels or intermittent viral replication will be missed. On the contrary, using highly sensitive assays (based on nucleic acid amplification), viremia can be detected also in HBV carriers without histologic evidence of liver disease; therefore, in such cases, the specific diagnosis of virus-induced liver disease can be missed.
2. IgM anti-HCV detection. The sensitivity and specificity of the assay are lower as compared to the assays currently used for IgM anti-HBc detection. We hope that the introduction of IgM capturing assays on the model of anti-HBc IgM will improve the sensitivity of the assay in the near future.
3. Nucleic acid detection. The assays that can be used for nucleic acid detection have an analytical sensitivity ranging from 10^6 to 10 genomes equivalent according to the method (direct probe hybridization or nucleic acid amplification). The clinical sensitivity is influenced by other factors such as the sample volume, the method used for nucleic acid purification, and the presence of inhibitors ***(52–55)***.
   The availability of highly standardized and authomatized protocols for both extraction and amplification procedures will significantly improve the reproducibility of the assay.
4. Quantitative analysis of silver-staining nucleolar organiser regions has been initially measured as the mean area occupied by AgNor and it was considered to be associated with the risk of developing HCC when larger than 3 μm^2 ***(32)***. More recently, the quantitation of the mean AgNor area has been associated with the new calculation of the proliferation index, defined as the percentage of hepatocytes with AgNor areas larger than 7 μm^2. The new analytical method appears to be more specific for the identification of cell proliferation and more reproducible as far as the interobservers' variations are concerned.

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7

Mutation of p53 Tumor Suppressor Gene in Hepatocellular Carcinoma

Apollonia Tullo and Elisabetta Sbisà

1. Introduction

In recent years, the most commonly observed genetic alteration in hepatocellular carcinoma (HCC), as in many other tumors affecting man, has been reported to be the mutation of the p53 coding gene ***(1,2)***. This gene has the features of a recessive oncosuppressor in its wild-type form and can be a dominant oncogene in its mutated form. The gene (20 kb) is located in a single copy on the short arm of chromosome 17 and contains 11 exons interrupted by 10 introns. The mRNA (2.8 kb) codes for a protein of 393 amino acids, which is expressed at relatively low levels in all tissues. p53 product is a 53-kDa phosphoprotein involved in the regulation of cell cycle, in DNA synthesis and repair, and in cell differentiation and apoptosis (see refs. ***3–6***, for reviews).

Different tumors show different mutation patterns as to mutation type and location. Furthermore, some mutations are typical of certain geographical areas (Asian countries) and depend on exposure to environmental mutagens (e.g., aflatoxin B1). The mutational spectrum of the p53 gene associated to a given tumor is important in understanding the etiology of cancer and, thus, it can be exploited to prognostic and therapeutic aims. Indeed, cancers presenting mutations of p53 tend to be more agressive, resist chemotherapy, and are less respondent to most protocols than cancers of similar tissues expressing wild-type p53 ***(1,7)***. Moreover, it has been reported that the replacement of the normal p53 oncosuppressor gene ***(8)*** inhibits growth and/or tumorigenicity in the tumoral cell lines of the colon ***(9)*** lung ***(10)*** and in HCC ***(11)***. For these reasons the coming together of complementary experiences such as those of

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

the biologist and of the clinician makes molecular diagnosis and therapy a revolutionary reality in future medicine offering the opportunity to develop new strategies in the treatment of cancers.

In our study on Mediterranean patients affected by HCC, fibrolamellar variant (FLC) and sarcomatoid transformation of HCC, we have performed the characterization of the p53 mutational pattern by single-strand conformation polymorphism (SSCP) and direct sequencing of all 11 exons ***(12)***, thus accounting also for exons 1–4 and 9–11 usually not reported in the literature. As described in this chapter, we optimized SSCP and sequencing conditions for all 11 exons thus solving, by appropriate measures, many of the difficulties created by the high guanine-cytosine (GC) content of the gene. This causes high- ''compression'' areas in the sequencing gel, which even automatic sequencing machines cannot cope with easily. The use of deazanucleotides, AmpliTaq DNA polymerase, and controlled-temperature conditions in the electrophoretic run have solved most of the problems and have resulted in unambiguous sequences that can be compared with those of normal individuals used as control. Our conditions allow optimization of yields, sensitivity, time, and costs for what could become a mass molecular screening.

The genomic DNA was extracted from nontumoral and tumoral tissues of HCC patients by the phenol extraction method ***(13)***. We have set up the conditions to amplify the complete p53 coding region, including exon/intron junctions by polymerase chain reaction (PCR). PCR is a powerful in vitro method of nucleic acid synthesis by which a particular segment of DNA can be specifically replicated. It involves the use of a pair of oligonucleotide primers that flank the target DNA fragment to be amplified. These primers hybridize to opposite strands of the target sequence and are oriented so that DNA synthesis by the polymerase enzyme proceeds across the region between the primers. Because the extension products themselves are also complementary to and capable of binding primers, repeated cycles of amplification essentially result in an exponential increase in the amount of the target DNA synthesis. Because the human p53 gene sequence was known ***(14)***, the amplification primers for the DNA amplification were appropriately selected from the human p53 gene sequence (Table 1).

The amplified products are purified to eliminate primers and nonspecific products and analyzed by single-strand conformation polymorphism (SSCP). SSCP is a technique devised to permit rapid screening for unknown mutations by taking advantage of the fact that single-nucleotide changes in DNA sequence alter the mobility of single-stranded DNA in nondenaturing gels ***(15,16)***. The PCR-amplified products can be separated into single strands by denaturation and electrophoresed on polyacrylamide gels under denaturing conditions. The rate of migration through the gel is a function of conformation, which, in turn,

Table 1
p53 Primers Used in PCR Amplification and Cycle Sequencing Experiments

Exon	Forward Primer 5'–3'	Position	Reverse Primer 5'–3'	Position	Length (nt)
1	TTTCCCCTCCCATGTGCTC	790–808	AAAATACACGGAGCCGAGAG	1013–994	224
2	AAGCGTCTCATGCTGGATCC	11,651–11,670	CAAGAGCAGAAAGTCAGTCC	11,897–11,878	247
3	AGCAGAGACCTGTGGGAAGC	11,847–11,866	CATCCATTGCTTGGGACGGC	12,048–12,029	202
4	GCTGGGGGGCTGAGGACCTG	11,969–11,988	ATACGGCCAGGCATTGAAGTCTC	12,351–12,229	383
5	TGTTCACTTGTGCCCTGACT	13,005–13,024	AGCAATCAGTGAGGAATCAGAG	13,314–13,293	310
6	AGAGACGACAGGGCTGGTTG	13,257–13,276	GAGGGCCACTGACAACCACC	13,492–13,473	236
7	TGCTTGCCACAGGTCTCCCCAA	13,939–13,960	TCAGCGGCAAGCAGAGGCTGG	14,172–14,152	234
8	GACCTGATTTCCTTACTGCC	14,403–14,422	AGGCATAACTGCACCCTTGG	14,641–14,622	239
9	CCAAGGGTGCAGTTATGCCT	14,622–14,641	GCATTTTGAGTGTTAGACTGG	14,813–14,793	192
10	AACCATCTTTTAACTCAGGTAC	17,510–17,531	AATCCTATGGCTTTCCAACCT	17,744–17,724	234
11	GGCACAGACCCTCTCACTCATG	18,540–18,561	TTGCAAGCAAGGGTTCAAAGACC	18,776–18,754	237

Note : The position number refers to the p53 human sequence (EMBL Accession number X54156).

depends on base sequence. Therefore, a mutation may alter the rate of migration of one or both single strands with respect to a normal exon (Fig. 1). The sensitivity of this approach increases with smaller fragment size and does not detect all mutations.

The optimal separation of the two strands for each exon is obtained by varying the concentration of the polymers in the gel (acrylamide and bisacrylamide), gel composition with glycerol, and changing the running time. After running, gel is silver stained.

Exons showing an altered migration are sequenced to define the nature of the nucleotide change. The mutation has to be confirmed on the two strands by sequencing both with forward and with reverse primers. The main sequencing technique in current use is the enzymatic method of Sanger. This method is based on the use of specific terminators of DNA chain elongation: 2',3'-dideoxynucleoside-5'-triphosphates (ddNTP). These deoxynucleoside triphosphates analogues can be incorporated by a DNA polymerase into a growing DNA chain through their 5'-triphosphate groups. However, because these analogues lack hydroxyl group at the 3'-position, they cannot form phosphodiester bonds with the next incoming deoxynucleoside-5'-triphosphates (dNTP), and the chain extension terminates whenever an analogue is incorporated. Thus, when a specific ddNTP is included along with the four dNTP normally required for DNA synthesis, the resulting extension products are a series of discrete-length DNA chains that are specifically terminated at the dideoxy residue. To obtain sequence data, one extension reaction must be run for each of the four ddNTPs. When analyzed individually, these reactions give specific chain-termination data; collectively, the data provide complete sequence information (Fig. 2). The cycle sequencing method we used takes advantage of the Sanger procedure combined with the amplification offered by multiple rounds of PCR ***(17)***.

In those cases when the mutation is contained in a restriction site, restriction enzyme analysis is a fast method for screening known point mutation. Restriction endonucleases are enzymes, isolated chiefly from prokaryotes, that recognize specific sequences within double-stranded DNA. Gain or loss of a restriction site may result in a change in the length of restriction fragments generated with a known enzyme (e.g., TaqI identifies p53 variants at codon 213 [Fig.3] HaeIII at codon 249, CfoI at codons 158 and 175).

The detected mutations can be searched against the p53 specialized database ***(18)*** through the Internet, allowing the comparison of results obtained against those collected in the database. It is thus possible to assess if the sequence variations detected can be associated to a pathology.

The p53 mutations are then correlated with the clinical profile of each patient. In our study ***(12)***, no mutation was detected in FLCs, HCCs without

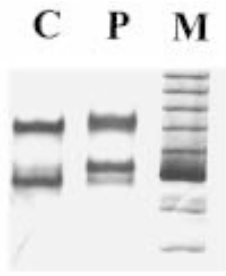

Fig. 1 Exon 7 PCR product SSCP analyses. C=control, P=patient, M=size marker. The type of mutation is assessed by sequencing (Fig. 2).

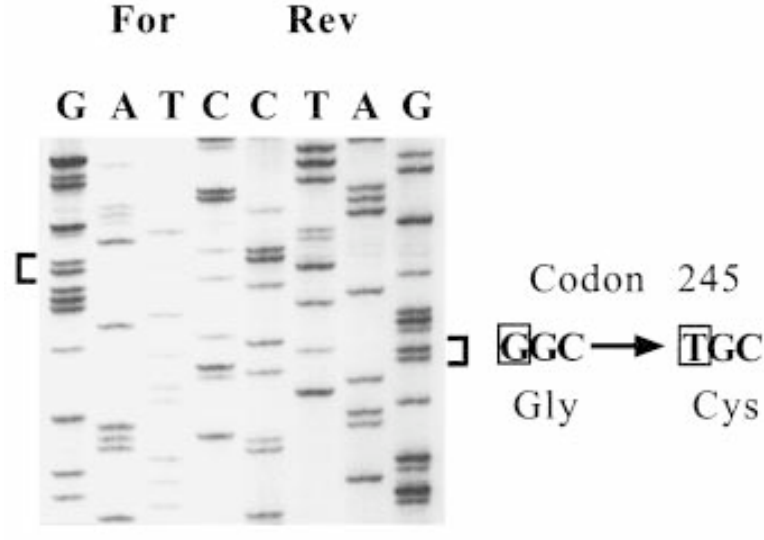

Fig. 2. Direct sequencing of exon 7. Tumoral DNA missense mutation in codon 245 verified on both strands with forward (For) and reverse (Rev) primers

cirrhosis, and HCCs with alcoholic cirrhosis. The p53 mutations occurred mainly in the group of HCC patients with liver cirrhosis associated with viral hepatitis. The two HCC patients with sarcomatoid changes had mutations in the gene. Survival data following liver resection showed that the mean and median survival times in patients with wild-type p53 to be 60 and 43 mo, respectively. In the group with p53 mutation, the mean and median survival was 15 and 12 mo ($p = 0.0034$). Likewise in our study we found p53 to be a poor prognostic indicator for survival in patients undergoing liver resection. Future studies might show that tumor staging might include the preoperative determination of p53 status in these patients.

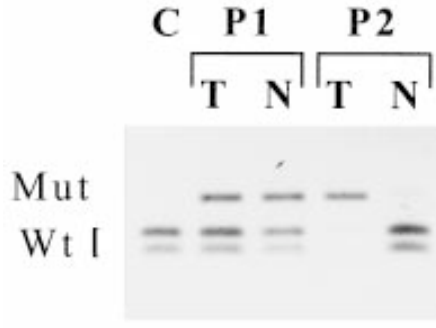

Fig. 3. Exon 6 mutations: codon 213 restriction analysis. *Taq*I restricted exon 6 PCR products (236bp) of patients (P) 1 and 2; C=Control, T=tumoral, N=nontumoral. *Taq*I digests the wild type (wt) DNA into two fragments of 140 and 96 bp. Substitution of any bases in the codon 213 determines the loss of the restriction site. Patient 2 has an homozygous mutation in tumoral DNA. The type of mutation is assessed by sequencing.

The presence of p53 mutation might discourage surgeons from considering resection and might be a favorable criteria for inclusion in a gene therapy program.

2. Materials

When preparing solutions, use sterile deionized, distilled water and reagents of the highest grade available. Sterilization is recommended for most applications and is, in general, accomplished by autoclaving. It is important to follow laboratory safety guidelines and follow manufacturers' precautions when working with hazardous chemicals ***(19)***. Most simple stock solutions can be stored at room temperature; when more rigorous conditions are required, this is indicated in the individual recipies.

2.1. Sample Collection and Purification of Genomic DNA from Tissues

1. DNA to be purified (2–3 g).
2. Tissue buffer: 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM EDTA.
3. 10 mg/mL Proteinase K in 0.075M NaCl, 0.024M EDTA pH 7.5.
4. 10% sodium dodecyl sulfate (SDS).
5. 3M CH_3COONa pH 7.0.
6. TE-buffered phenol pH 8. Store at 4°C.
7. TE-buffered phenol pH 8/chloroform (1 : 1). Store in brown glass bottle <2 mo at 4°C.
8. 95% ethanol (EtOH), ice cold.
9. 70% Ethanol, room temperature.
10. 1X TE buffer: 10 mM Tris–HCl pH 8.0, 1 mM EDTA.

11. 15mL polypropylene sterile centrifuge tubes.
12. Mortar and pestle.
13. Liquid nitrogen.
14. Centrifuge.
15. 37°C water bath.
16. Sterile glass rod.

Caution: Human tissues, chloroform, and phenol are hazardous.

2.2. Polymerase Chain Reaction

1. Genomic DNA (500 ng).
2. Oligonucleotide primers: reverse and forward (Table 1).
3. 1.25 mM dNTP mix: 125 µL of 10 mM dATP, 125 µL of 10 mM dCTP, 125 µL of 10 mM dTTP, 125 µL of 10 mM dGTP, sterile water to 1 mL.
4. Taq DNA polymerase.
5. 10X Taq amplification buffer supplied by the manufacturer.
6. 1.5 and 0.2 mL polypropylene sterile microcentrifuge tubes.

2.3. Purification of Amplified PCR Products

2.3.1. Agarose Gel

1. Agarose, electrophoresis grade.
2. 10X TBE buffer (pH 8.3): 108 g Tris base (0.89M), 54 g boric acid (0.87 M), 1.86 g EDTA (0.005 M), water to 1 L. Prepare weekly.
3. DNA molecular size marker.
4. Ethidium bromide solution (10 mg/mL): Dissolve 0.2 g ethidium bromide in 20 mL water. Mix well and store at 4°C in dark bottle. Ethidium bromide is a powerful mutagen. Always wear gloves while handling gels or solutions containing the dye.
5. 6X loading buffer: 0.25% Bromophenol Blue, 0.25% xylene cyanol, 30% glycerol in water. Store at 4°C.
6. Gel casting platform, gel comb.
7. Horizontal gel electrophoresis apparatus.
8. Power supply.
9. Ultraviolet (UV) transilluminator. Caution: Ultraviolet light can damage eyes and skin. Wear UV-blocking goggles or a face shield and minimize time spent viewing.

2.3.2. DNA Elution

1. QIAquick Gel Extraction Kit (Qiagen).
2. 1.5 polypropylene microcentrifuge tubes.
3. Microcentrifuge.

2.4. Single-Strand Conformation Polymorphism

1. PCR product (300 ng).
2. 1% sodium deoxycolate.

3. 0.11M EDTA pH 7.5.
4. Loading buffer: 98% deionized formamide, 0.3% Bromophenol Blue/0.3% xylene cyanol. Caution: Formamide is hazardous.
5. 5X TBE buffer (pH 7.5): 54.5 g TRIS (0.45M), 55 g boric acid (0.89M), 5.85 g EDTA (0.016M), water to 1 L.
6. Acrylamide–bisacrylamide stock solution 25% (49 : 1): acrylamide 24.5 g, bisacrylamide 0.5 g, distilled water to 100 mL. Heat the ingredients at 55°C in a beaker while stirring. When dissolved, add water to 100 mL total volume. Filter solution through Whatman 3MM filter paper. Store for up 1 wk at 4°C in a dark bottle. Caution: Acrylamide is neurotoxic. Wear gloves and mask when preparing acrylamide reagents.
7. Glycerol.
8. 10% (w/v) APS (ammonium persulfate). Make fresh weekly and store at 4°C.
9. TEMED (N,N,N',N'-tetrametiletilendiammina). Caution: TEMED is hazardous.
10. 90°C heating block.
11. 8 × 10 cm, 0.75-mm thick polyacrylamide gels.
12. Slab–mini-gel apparatus.
13. Power supply.

2.5. Silver Staining

1. 10% ethanol.
2. 1% HNO_3.
3. Staining solution: 0.012 M $AgNO_3$; dissolve 0.2 g $AgNO_3$ in 100 mL distilled water. Silver nitrate is light sensitive so the solution is stored in a dark bottle at room temperature. Caution: Silver nitrate powder is hazardous.
4. Developing solution: 0.28M Na_2CO_3/0.019% formaldehyde; dissolve 3g sodium carbonate (MW 105.99), and 51 µL 37% formaldehyde, distilled water, and make up to 100 mL. Prepare fresh.
5. 2% acetic acid.
6. Whatman 3MM filter paper.
7. Gel dryer.

2.6. DNA Sequencing

2.6.1 Cycle Sequencing

1. Template DNA (50 ng).
2. Oligonucleotide primer.
3. AmpliCycle Sequencing Kit (Perkin Elmer). Reagents provided:
   10X cycling mix: AmpliTaq DNA Polymerase, CS, in 500 mM Tris–HCl, pH 8.9, 100 mM KCl, 25 mM $MgCl_2$, 0.25% (v/v) Tween-20
   G Termination mix: 22.5 µM c7dGTP, 10 µM each dATP, dCTP, and dTTP and 80 µM ddGTP in 10 mM Tris–HCl, 0.1 mM EDTA, pH 7.5
   A Termination mix: 22.5 µM c7dGTP, 10 µM each dATP, dCTP, and dTTP and 600 µM ddATP in 10 mM Tris–HCl, 0.1 mM EDTA, pH 7.5
   T Termination mix: 22.5 µM c7dGTP, 10 µM each dATP, dCTP, and dTTP and

900 µM ddTTP in 10 mM Tris–HCl, 0.1 mM EDTA, pH 7.5
C Termination mix: 22.5 µM c7dGTP, 10 µM each dATP, dCTP, and dTTP and 300 µM ddTP in 10 mM Tris–HCl, 0.1 mM EDTA, pH 7.5
Stop solution: 95% Formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.02% xylene cyanole FF. Formamide is a teratogen. Wear gloves.

4. 1.5 and 0.2 mL polypropylene sterile microcentrifuge tubes.
5. α^{35}S-dATP, 10 mCi/mL, 1000 Ci/mmol.
6. Mineral oil.

Store according to manufacturer's instructions. Use within 45 d of calibration date. Caution: Radioactive substances are hazardous and require special handling.

2.6.2. Denaturing Gel Electrophoresis for Sequencing

1. 6% acrylamide – 7M urea stock solution: 57 g acrylamide, 3 g bisacrylamide, 420.5 g urea (ultrapure), 100 mL of 10X TBE (pH 8.3), add water to 1 L. Heat the ingredients at 55°C in a beaker while stirring. When dissolved, add water to 1 L total volume. Deionize by stirring with 5 g Amberlite MB-1 (Sigma) 30 min at room temperature. Filter solution through Whatman 3MM filter paper. Store 2–4 wk at 4°C in a dark bottle.
2. Repel-Silane.
3. Binding-Silane stock: 50 mL ethanol 95%, 150 µL γ-metacrilossipropiltrimetoxilane.
4. 10% acetic acid.
   Caution: Repel-Silane and Binding-Silane are toxic and highly volatile and should be used in a fume hood. Wear gloves during siliconization and avoid inhaling fumes.
5. Gel fixation solution: 10% acetic acid, 12% methanol.
6. 10% APS.
7. TEMED.
8. 10X TBE buffer (pH 8.3): 108 g TRIS (0.89M), 54 g boric acid (0.87M), 1.86 g EDTA (0.005M), add water to 1 L. Prepare weekly.
9. Termal cycler.
10. 90°C heating block.
11. Sequencing apparatus with a thermoplate to ensure even heat distribution across the gel surface, glass plates (20 × 40 cm), spacers (0.2 mm), tooth comb.
12. Power supply capable of delivering a minimum of 100 constant watts.
13. Film cassette (35 × 43 cm).
14. X-ray Film, Kodak BioMax Film, 20 × 40 cm
15. Film processing equipment.

2.7. Restriction Analysis

1. PCR-amplified exons (300–500 ng).
2. Restriction enzyme.
3. 10X restriction buffer provided by manufacturer.
4. Agarose gel (see Subheading 2.3.1.).

5. Water bath.
6. Polaroid camera with orange filter, UV-blocking filter.

2.8. Data Analysis

p53 Specialized database, available at IARC (http://www.iarc.fr/p53/homepage.htm) or from the EBI server (http://www.ebi.ac.uk) ***(18)***.

3. Methods

Characterization of p53 coding regions. The following methods are applied to screen tissues of the selected patients.

3.1. Sample Collection and Purification of Genomic DNA from Tissues

The tumoral and nontumoral tissues of patients after surgery are immediately snap-frozen in liquid nitrogen and stored at -80°C.

1. Pour liquid nitrogen into a mortar and reduce the tissue (2–3 g) to a fine powder with a pestle. Let the liquid nitrogen evaporate and transfer the crushed tissue into a sterile Falcon tube on ice.
2. Suspend the powder in 5 mL of cold tissue buffer and add 50 µL of Proteinase K, 10 mg/mL (cf. 100 µg/mL) and 250 µL 10% SDS (cf. 0.5%).
3. Incubate overnight at 37°C under shaking.
4. Extract the digested DNA samples with 1/10 volume 3M CH_3COONa pH 7.0 and an equal volume of phenol/chloroform (1 : 1).
5. Mix gently for 15 min at room temperature.
6. Centrifuge for 3 min at 800g at room temperature.
7. Carefully remove the upper aqueous phase containing the DNA and transfer to a fresh tube and repeate the extraction (steps 4 – 6).
8. Transfer the upper aqueous phase to a fresh tube and recover the DNA by precipitation with 2 volumes of ice-cold 95% EtOH. Mix well.
9. Wind out the DNA clot with a sterile glass rod.
10. Wash the DNA pellet with a solution of 70% EtOH.
11. Drain off as much supernatant as possible and dry the pellet at room temperature.
12. Dissolve the pellet in 1 mL of sterile water or in 1X TE buffer (see Note 1).
13. Store the DNA at 4°C.
14. Quantify the DNA by measuring the absorbance at 260 nm UV, in a spectrophotometer.

3.2. Polymerase Chain Reaction

Amplification of contaminating sequences is a potential problem with PCR. To minimize this risk, filtered pipet systems should be used and reagents and equipment should be segregated from general lab use. Wear disposable gloves. Use only sterile disposable plasticware and cotton-plugged tips to minimize the transfer of DNA by aerosol.

For each sample, set up the following 100 µL reaction in a 0.2-mL polypropylene microcentrifuge tubes (see Note 2):

Table 2
p53 Exon Amplification Conditions

Exon	Each of 35 Cycles				
	Initial Step	Melt	Annealing	Extension	Final Step
1	5 min	50 s	50 s	45 s	2 min
	94°C	94°C	58°C	72°C	72 °C
2	5 min	50 s	50 s	50 s	2 min
	94°C	94°C	58°C	72°C	72 °C
3	5 min	50 s	45 s	45 s	2 min
	94°C	94°C	63°C	72°C	72°C
4	5 min	50 s	50 s	60 s	2 min
	94°C	94°C	67°C	72°C	72°C
5	5 min	50 s	50 s	60 s	2 min
	94°C	94°C	59°C	72°C	72°C
6	5 min	50 s	50 s	45 s	2 min
	94°C	94°C	63°C	72°C	72°C
7	5 min	50 s	50 s	45 s	2 min
	94°C	94°C	68°C	72°C	72°C
8	5 min	50 s	50 s	45 s	2 min
	94°C	94°C	58°C	72°C	72°C
9	5 min	50 s	50 s	45 s	2 min
	94°C	94°C	56°C	72°C	72°C
10	5 min	50 s	50 s	45 s	2 min
	94°C	94°C	58°C	72°C	72°C
11	5 min	50 s	50 s	45 s	2 min
	94°C	94°C	65°C	72°C	72°C

500 ng of genomic DNA.
30 pmol of appropriate oligonucleotide primer pairs (Table 1) (see Note 3).
16 µL 1.25 mM 4dNTP (0.2 mM).
10 µL of 10X Taq amplification buffer supplied by the manufacturer.
2.5 U of Taq DNA polymerase.
Sterile water to 100 µL.

Carry out PCR using the amplification cycles we optimized for each exon (Table 2).

3.2.1. Purification of Amplified PCR Products

Purify the amplified products to eliminate primers and nonspecific products on 1.5% agarose TBE gel stained with ethidium bromide. Include a lane that contains a known quantity of molecular size standard. The appropriate band is cut out from the gel and eluted using QIAquick Gel Extraction kit (Qiagen) with the following protocol.

3.2.1.1. Agarose Gel

1. Add 1.5 g of powdered agarose to 100 mL of 1X TBE.
2. Heat the slurry in a microwave oven until the agarose dissolves.
3. Cool the solution to about 50°C and add ethidium bromide (cf. 0.5 µg/mL).
4. Pour the melted solution into the gel caster (12 × 12 cm), place the comb and allow the gel to polymerize for 30–45 min at room temperature.
5. Carefully remove the comb, place the gel in the electrophoresis tank and add just enough electrophoresis buffer 1X TBE to cover the gel.
6. Add 1/10 loading buffer to the DNA sample (1/10 PCR amplification reaction) and load into the wells (see Note 4).
7. Run the gel at 100 V for 2 h. The negative lead is attached to the end of the box nearest the gel wells, as DNA will move toward the positive electrode.
8. Examine the gel under UV illumination (see Note 5).

3.2.1.2. DNA Elution

Use the QIAquick Gel Extraction Kit (Qiagen).

1. Excise the DNA fragment from the agarose gel with a sterile scalpel, transfer the gel slice in Eppendorf tube and weigh.
2. Add 300 µL of Buffer QX1 (supplied by the manufacturer) for each 100 mg of gel.
3. Incubate at 50°C for 10 min. To dissolve gel, mix the tube two to three times.
4. To bind DNA, load the completely dissolved gel slice to a QIAquick spin column, placed in the collection tube (the maximum volume of the column is 800 µL; for sample volumes of more than 800 µL, load and spin again).
5. Centrifuge for 1 min in Eppendorf centrifuge at 13,600 g.
6. Discard the flowthrough and place the column back into the same tube.
7. To wash, add 750 µL of buffer PE (supplied by the manufacturer) to the column, wait for 5 min, and centrifuge for 1 min at 13,600 g.
8. Discard the flowthrough and centrifuge again for 1 min to eliminate residual ethanol of buffer PE.
9. Place the column into a sterile Eppendorf tube and add 40 µL of sterile water and wait for 1 min to elute the DNA
10. Centrifuge for 1 min at 13,600 g.
11. To quantify the DNA, load 1/10 eluted DNA on 1.5% agarose gel including a lane that contains a known quantity of molecular size marker.

3.3. Single-Strand Conformation Polymorphism

The ability to detect mutations by SSCP decreases for longer PCR products; therefore, PCR primers were selected, so only short fragments of DNA (200–300 bp) are amplified.

1. Prepare glass plates (8×10 cm) by cleaning throughly with detergent, water, and ethanol. Insert spacers (0.75mm thick) and seal the sides and bottom with waterproof tape (see Note 6).
2. Prepare gel solution as reported in Table 3 (see Note 7). For a 8% acrylamide–10% glycerol nondenaturing gel (8 mL), mix:

Table 3
SSCP Nondenaturing Gel Conditions

Exon	Acrylamide–Bisacrylamide (%)	Glycerol (%)	Xylene Cyanol Migration (cm)
1	8	10	12
2	8	10	10
3	9	10	12
4	6	10	10
5	8	10	14
6	8	–	14
7	9	10	10
8	7	5	11
9	6	–	15
10	8	10	15
11	8	–	12

   a. 2.6 mL 25% (49 : 1) polyacrylamide stock solution.
   b. 0.8 mL 5X TBE buffer pH 7.5.
   c. 1mL 80% glycerol.
   d. 3.6 mL distilled water.
   e. 80 µL 10% APS.
   f. 8 µL TEMED.
   Mix well.
3. Pour gel immediately. Remove any bubbles by tapping the plates. Insert square-tooth comb, clamp well and lay at least 45 min to polymerize.
4. Remove the clamps, the tape from the bottom of the gel, and the comb. Attach the plates to the electrophoresis apparatus. Add 0.5X TBE pH 7.5 to top and bottom tanks (see Note 8). Flush out the wells and the bubbles from the bottom of the gel using a syringe with a needle.
5. Mix 5 µL of PCR product (300 ng) with 2 µL of 1% sodium deoxycolate, 2 µL of 0.11 M EDTA pH 7.5, and 5 µL loading buffer. Boil the samples for 5 min, then place sample immediately on ice to prevent the DNA strands from reannealing.
6. Immediately load the samples on nondenaturing polyacrylamide gel. Include lanes with PCR-amplified positive and negative control DNA (see Note 9). Electrophorese 1–4 h depending on the fragment size and gel percentage at 200 V (Table 3). A constant internal buffer temperature (20°C) should be maintained during the gel run. Gels containing glycerol generally run more slowly.
7. After electrophoresis, disconnect power and remove plates from the apparatus. Remove tape from the sides and lay plates flat. Pry plates apart (the gel should stick to one plate) and silver stain the gels (Fig.1).

3.4. Silver Staining

Silver staining is a rapid, sensitive nonradioactive staining. Use 100 mL of each solution in the following steps:

1. Wash the gel for 7 min in 10% ethanol. Decant the solution.
2. Oxidize the gel 3 min in 1% nitric acid (HNO_3) under shaking. Decant the solution.
3. Rinse the gel 5 s in double-distilled water. Decant the water.
4. Add silver nitrate staining solution (0.012M $AgNO_3$) to the gel. Incubate for 20 min under shaking. Decant the silver nitrate solution.
5. Rinse the gel 5 s in double-distilled water. Decant the water.
6. Add 0.28M Na_2CO_3/0.019% formaldehyde developing solution to reduce the gel. Gently shake the gel. Decant the developing solution after the bands appear. The length of developing time varies with each gel. Avoid overdeveloping the gel. Decant the developing solution before the gel turns brown as a result of excess silver salts.
7. Stop the developing process by incubating the gel 2 min in 2% acetic acid. Decant the solution.
8. Rinse the gel 5 s in double-distilled water. Decant the water.
9. Transfer gel to a sheet of Whatman 3MM filter paper and cover with plastic wrap.
10. Vacuum dry the gel 30 min at 80°C.

3.5. DNA Sequencing

Exons showing an altered migration in SSCP should be sequenced on both strands to confirm the mutations (Fig. 2). Cycle sequencing is a fast and efficient method to screen for mutation fragments up to 300 bp in length. When the tumor is identified to have a mutation, the p53 sequence of the nontumorous liver tissue from the same individual should be also evaluated (see Note 10).

3.5.1. Cycle Sequencing

Use AmpliCycle Sequencing Kit (Perkin Elmer).

1. For each sample to be sequenced, prepare four thermal cycler tubes and add 2.5 µL of the G, A, T, C termination mixes; store tube on ice.
2. For each sample, prepare 30 µL of the reaction mix containing the following:
   Template DNA (50 ng) (*see* **Note 11**).
   20 µM of appropriate oligonucleotide primer (Table 1).
   10X cycling mix 4 µL.
   α^{35}SdATP (10 µCi/mmol).
   Sterile water to 30 µL.
3. Place 6.5 µL aliquots of reaction mix into each of the four cycle sequencing reaction tubes prepared in step 1.
4. Add mineral oil and start thermal cycling following the conditions in Table 1 except that the number of cycles is reduced to 25.

5. After completion of thermal cycling, add 4 µL stop solution to each reaction. Store sequencing reactions at -20°C for up to one week.
6. Analyze sequencing reaction products on a denaturing 6% polyacrylamide / 7M urea gel.

3.5.2. Denaturing Gel Electrophoresis for Sequencing

To facilitate the handling of large ultrathin gels, the gel is covalently bound to the notched glass plate using Bind-Silane (mix 2.5 mL Binding-Silane stock with 150 µL acetic acid 10%) and the thermostatic plate with Repel-Silane to ensure that the gel does not stick to this plate.

1. Clean matched glass plates with ethanol.
2. Using a lint-free tissue, spread Bind-Silane solution over the top surface of the plate.
3. Leave the plate to dry.
4. Rinse the plate with ethanol.
5. Using a lint-free tissue, spread Repel-Silane solution over the top surface of the thermostatic plate.
6. Leave the plate to dry.
7. Rinse the plate with ethanol.
8. Assemble the matched plates according to the manufacturer's instruction, using 0.2-mm spacers and clamps.
9. Prepare denaturing polyacrylamide gel solution by mixing 30 mL of stock solution, 150 µL 10% APS, and 15 µL TEMED.
10. Pour gel solution, avoiding bubbles formation and place the comb.
11. Allow the gel to polymerize for at least 1 h.
12. Remove the comb. Attach the plates to the electrophoresis apparatus. Add 1X TBE pH 8.3 to top and bottom tanks.
13. Preheat gel 30 min by setting power supply to 1700 V, 70 W constant power.
14. Rinse wells with 1X TBE buffer just prior to loading gels, using a syringe with a needle to remove urea that has leached into them.
15. Denature sequence reactions 5 min at 95°C, and cool rapidly on ice.
16. Load 2- to 3- µL sample per well of the four denatured reactions of one template into separate adjacent wells of the gel.
17. Run the gel at a constant wattage (2000 V) sufficient to maintain the gel at the desidered temperature (45–55°C) for the time required (2–4 h) to achieve optimal resolution of the sequence of interest (observe migration of marker dyes to determine length of electrophoresis).
18. After electrophoresis is complete, disconnect power, remove plates from the apparatus, and pry plates apart (the gel should stick to one plate).
19. Wash urea out of the gel with approximately 2 L of 10% acetic acid and 12% methanol for 20 min to avoid the film sticking to the gel.
20. Wash the gel with water.
21. Dry gel at 80°C for 1h.
22. Autoradiograph overnight at room temperature.
23. Develop the film according to the manufacturer's instructions.

3.6. Restriction Analysis

1. Digest purified PCR product (300–500 ng) with 5 U of the selected restriction enzyme and 1 μL of appropriate restriction buffer in 10 μL of reaction (see Note 12).
2. Incubate at the appropriate temperature (depending on the restriction enzyme used) for 2 h.
3. Add 1 μL of 6X loading buffer.
4. Load the digested DNAs on a 1.5% agarose gel (see Note 4). Include a lane for the molecular size marker.
5. Examine the gel under UV illumination and photograph (Fig. 3).

3.7. Data Analysis

To obtain the database, access the IARC p53 database website using the URL http://www.iarc.fr/p53/homepage.htm, selecting ''obtain the database'' and follow on-screen instructions. The database may be also obtained from EBI by anonymous ftp to ftp.ebi.ac.uk, in the directory/pub/database/p53 or by World Wide Web access using the URL http://www.ebi.ac.uk/, selecting ''services,'' going to the database selection and selecting ''IARC p53.''

The database contains over 6800 published mutations of the p53 gene in human cancers and tumoral cell lines.

4. Notes

1. Most pellets will be resuspended within 15 – 30 min, but samples may be resuspended overnight at room temperature. If the pellet is not in solution after this time, add more TE buffer. The DNA solution must be homogeneous. Aqueous DNA may be stored indefinitely at 4°C.
2. In each set of PCR reactants, a negative no-template control was included and processed in parallel to localize any contamination that does occur. For multiple samples, prepare a cocktail of all the reaction components (including the polymerase) and aliquot into each microcentrifuge tube containing sample DNA.
3. We synthesized amplification primers for each exon. Primers for amplification and sequencing meet the following criteria:
   * to be at distant enough positions from 5' and 3' ends of exons, in order to be able to analyze also exon/intron junction regions likely to be involved in mutations critical for splicing
   * Each pair amplifying an exon to have a Tm common to several pairs so that in a single PCR_1 run different exons from a single patient can be examined and thus screening time can be speeded up
4. The maximum amount of DNA that can be applied to a slot depends on the number of fragments in the sample and their size. The minimum amount of DNA that can be detected by photography of ethidium bromide-stained gels is about 5 ng in a 0.5-cm-wide band. If there is more than 200 ng of DNA in a band of this width, the slot will be overloaded.

5. Prolonged exposure of DNA to UV light should be avoided because this can introduce nicks to the DNA.
6. It is important that the gel be of uniform thickness. Support the plates at the sides and in the middle to help ensure uniformity.
7. Glycerol may be included in the gel mix to improve resolution. Higher concentrations of acrylamide may also be used. Variation in glycerol content and acrylamide concentration can dramatically affect the mobility and separation of the molecules. No denaturant, such a urea, should be used.
8. Use of 0.5X TBE buffer appears to give slightly better results than 1X TBE.
9. The gels are not always easy to interpret because of poor resolution and the presence of additional bands resulting from alternative conformations in unmutated sequences. A normal control DNA and undenatured DNA should be included to identify which bands are derived from a normal sequence and which may arise from partially denatured DNA.
10. When the sequencing gels, the SSCP, and the restriction analyses revealed both the mutant as well as the wild-type bands, these tumors may have a p53 mutation but not deletion on the remaining wild-type allele, alternatively, the wild-type band may be derived from the contamination of tumor DNA by nontumoral stroma liver DNA.
    When the mutated samples show a single mutant band, compared to controls, they most probably represent a homozygous state resulting from the pairing of a mutant allele with a deletion in the remaining allele.
11. The quality of the DNA template determines the success of a reaction. Care must be taken to ensure that the amplified PCR products consists of a single DNA fragment and free from oligonucleotide primers and excess deoxynucleotides. The template/primer ratio is also important.
12. The amount of DNA that can be digested depends on the number of fragments expected in the sample and their size.

Acknowledgment

The work has been supported by Italian Association for Cancer Research (AIRC).

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8

Diagnostic Markers in Hepatocellular Carcinoma Using Immunohistochemical Techniques

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1. Introduction

Hepatocellular carcinoma (HCC) is the seventh most common cancer in men and the ninth in women with an estimated incidence of about 1 million per year worldwide. HCC also accounts for 90% of all primary hepatic malignancies, and in most cases, appears to be a consequence of chronic infection of the liver by hepatotropic viruses (hepatitis B and hepatitis C viruses) ***(1)***. It is a highly malignant tumor with a poor prognosis that has been attributed to late diagnosis. Detection of HCC at an early stage may result in more effective treatment. However, the lack of symptoms in the early stage of the disease makes screening of patients at risk for HCC impractical. Surgical tumor resection or liver transplantation has been accepted as the only means of cure, but the postoperative recurrence within the remaining liver or even in the transplanted organ is also a cause of poor prognosis ***(2)***. It is therefore important to identify factors in tissues that can predict tumor recurrence and prognosis after resection in order to provide adjuvant therapy to different patient groups. It has recently been reported that proliferation rate and markers of cell loss, such as necrosis and apoptosis, may have prognostic value. Proliferation markers such as MIB-1 (Ki-67) and proliferating cell nuclear antigen (PCNA) have been studied in tumor specimens using immunohistochemical techniques and showed considerable tumor heterogeneity ***(3)***. α-Fetoprotein, an oncofetal protein produced by HCC, does not appear to play an important role in the diagnosis of advanced HCC; therefore, its use is limited because at least one-third of small HCC and 10%

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

of the advanced HCC will be missed ***(4)***. These limitations have motivated many investigators to search for other tumor markers for HCC, including transforming growth factor-β_1 (TGF-β_1), whose overexpression has been reported in transformed cells, tissue, and plasma of human HCC. The demonstration of normal synthesized proteins such as albumin, fibrinogen, α_1-antitrypsin, and chymotrypsin is also useful in identifying the tumor as being of liver cell origin ***(5)***. Major histocompatibility complex (MHC) antigens are expressed at very low levels by normal hepatocytes, but Class 1 and 2 antigens are present in high levels on most liver carcinoma cells ***(6)***. Tumor markers CA 19-9 and CA 50 have been suggested as useful markers in the differential diagnosis of liver cell from bile duct carcinoma ***(7)***. In addition to this, a strong expression of transferrin receptor has been found in HCC and hepatoblastoma but not in benign liver tumors or hyperplastic nodules ***(8)***.

In this chapter, we describe a specific method of immunohistochemistry in diagnosing hepatocellular carcinoma or localizing an antigen in a biopsy specimen or in a fine-needle aspirate (FNA). Further reading about immunohistochemistry is provided in **ref. *9***.

Immunohistochemistry is a powerful technique that allows the demonstration of cell antigens in its tissue or cellular location, thus providing an opportunity to examine simultaneously both pathogenetic mechanism and their pathological consequences. It is therefore defined as the use of labeled antibodies as specific reagents for localization of tissue constituents (antigens) in situ ***(9)***. However, the success of the technique is dependent on both the preservation of the antigens and the type of antibody used (i.e., an antibody with high affinity with its antigen). There is inevitably some antigen degradation by endogenous proteases or by fixative and processing procedures. Formalin and glutaraldehyde fixatives are crosslinking fixatives that form links (hydroxy–methylene bridges) between reactive groups of adjacent protein chains. Fixed proteins can retain their antigenicity only if the crosslinking does not affect the amino acid sequences that bind to the antibody. Where sufficient material is available, a representative should be snap-frozen in liquid nitrogen, because frozen sections remain the most satisfactory technique for optimal demonstration of the widest range of antigens. For FNA specimens, centrifuge the sample for about 10 min and resuspend the pellet with a small amount of phosphate-buffered saline (PBS). Using a cytospin machine (30 *g*, 4 min), spin the cells onto a polysine-coated slide, and quickly fix the cells in absolute alcohol or acetone. In order for the immunohistochemical reaction to be seen in the microscope, a component of the reaction must carry a label. Different labels are used, including flourescent compounds, radioactive labels, and enzymes (e.g., peroxidase) ***(9)***.

2. Materials

2.1. Tissues

Formalin-fixed paraffin-embedded tissue sections, frozen tissue sections (particularly for flourescent antibody labels) or FNA.

2.2. Buffers

1. Phosphate-buffered normal saline (PBS):
   a. 8.7 g sodium chloride.
   b. 0.272 g potassium dihydrogen phosphate.
   c. 1.136 g Disodium hydrogen phosphate.

   Dissolve all salts separately in water, then mix, make up to 1L, and check pH is 7.2 – 7.4.
2. 0.05*M* Tris-buffered normal saline (TBS), pH 7.6:
   a. 6.07 g Tris(hydroxymethyl)methylamine.
   b. 8.7 g Sodium chloride.
   c. Concentrated hydrochloric acid

   Dissolve the Tris and sodium chloride in 900 mL water. Add concentrated HCl until the pH reaches 7.6. Make up to 1 L with water.

2.3. Other Reagents

1. Antibody diluent: PBS or TBS containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide, for long-term storage of antibody at working dilution at 4°C.
2. Hydrogen peroxide; stock solution: 30% aqueous (w/v), stored at 4°C.
3. 70% methanol in PBS, for cryostat sections.
4. Trypsin (make fresh as required):
   a. Trypsin (crude porcine), approximately 435 USP units/mg.
   b. Calcium chloride, 0.1% in water or 0.005*M* Tris–HCl buffer or Tris–HCl- buffered 0.9% sodium chloride (TBS), pH 7.6–8.0.
   c. 0.1*M* (0.4%) sodium hydroxide (aq.)
5. 0.01 *M* citrate buffer pH 6.0:
   a. 1.05 g citric acid
   b. 2*M* (8% aq.) NaOH.

   Dissolve citric acid in 450 mL distilled water, and NaOH until pH 6.0, then top up to 500 mL.
6. Antibodies and other immunoreagents.
   a. Normal serum (goat, swine, rabbit), for blocking.
   b. Primary antibody (monoclonal or polyclonal).
   c. Secondary antibody: biotinylated (goat anti-mouse, swine anti-rabbit, etc.).
   d. Third layer: peroxidase conjugated streptavidin.
7. Diaminobenzidine tetrahydrochloride (DAB). *Caution:* Carcinogenic, wear pair of gloves.

To make a stock solution, take 5 g of DAB and add 100 ml distilled water. Using a magnetic stirrer, stir for 20 min in a fume hood until completely dissolved; then aliquot into small tubes for storage at –20°C (*see* **Subheading 3.**).

3. Methods

In this section, we describe in detail the procedures for carrying out the highly sensitive peroxidase-labeled streptavidin method for the localization of antigen in tissue blocks or FNA. Streptavidin is a protein with four binding sites per molecule for a low-molecular-weight vitamin called biotin ***(9)***. In this method, the second antibody is biotinylated and the third reagent is streptavidin labeled with peroxidase.

3.1. Dewaxing of Paraffin-embedded Tissue Sections (Hydration)

Place tissue sections in three changes of xylene, absolute alcohol, and 70% alcohol, leaving them for about 1 min, then in tap water.

3.2. Blocking Endogenous Peroxidase

1. Dewaxed sections are placed in 0.6% hydrogen peroxide in tap water (2 mL of 30% H_2O_2 per 100 mL water) for 15 minutes. (This step can be performed either before or after the antigen retrieving step) (*see* **Subheading 3.3.2.**).
   For acetone-fixed air-dried frozen and FNA sections, endogenous peroxidase is blocked by a milder method, using 0.3% H_2O_2 in 70% methanol in PBS (1 mL of 30% H_2O_2 in 100 mL of 70% methanol in PBS) for 30 min at room temperature (RT).
2. Rinse sections briefly in water after the blocking.

3.3. Antigen Retrieval Procedure

This step is only used for formalin-fixed paraffin-embedded tissue sections in which antigenicity has been reduced by the formation of hydroxy–methylene bridges between components of the amino acid chains of proteins.

Heat-mediated or enzyme digestion antigen retrieval methods may be used, depending on the type of antigens to be localized.

3.3.1. Enzyme Digestion Antigen Retreival

Trypsin is routinely used for the digestion of proteins (*see* **Note 1**) such as:

Cytokeratin and other keratins
Factor VIII-related antigens
CD3, kappa, lambda collagens

1. The trypsin is dissolved in calcium chloride to make a 0.1% solution, and the pH is quickly adjusted to 7.8 with 0.1*M* sodium hydroxide (*see* **Subheading 2.3.**).
2. Immerse slides in the trypsin solution and place at 37°C for about 10–20 min.
3. Rinse the slides well in running tap water and proceed with immunostaining.

3.3.2. Heat-Mediated (Microwave) Antigen Retrieval Method

Some antigens previously unreactive in formalin-fixed paraffin-embedded tissue, even after protease digestion, could be retrieved by heating sections in a solution of a heavy metal salt in a microwave oven without affecting the structure of the tissue. The heavy metal was later replaced by simple buffers such as citrate buffer at pH 6.0. It was suggested that the heating provides the energy to rupture the hydroxyl bonds formed by the fixative with protein antigen, and also releases tissue-bound calcium ions, which contribute to tighter bonds with the fixative.

1. Place 300 mL of citrate buffer pH 6.0 in a plastic container, and in a separate plastic beaker, place about 200 mL distilled water.
2. Place both containers in a microwave oven and warm them for 2 min at about 750 W.
3. The sections are placed on a plastic slide rack.
4. At the end of the 2 min, remove the container of distilled water and place the plastic slide rack in the warm citrate buffer.
5. Heat for 20 min or less, depending on the type of antigen to be retrieved, checking the level of buffer every 5 min. Top off with the prewarmed distilled water if necessary.
6. At the end of the microwaving place container with the slides under running cold water to cool the slides, then proceed to immunostaining. (*See* **Note 2.**)

3.4. Immunostaining by Hand

1. Drain the slides and wipe them dry except for the area of the sections, which should stay moist with PBS. If required, a water-repellent circle can be drawn around the sections after the antigen retrieval procedure, using the "DAKO pen." (This is not done to slides put on the sequenza, as it impedes flow of fluid to the sections.)
2. Lay the slides in a Petri dish on a rack made from two applicator sticks, containing some paper tissue well damped with water to provide a humidify chamber. Ensure sections are not dry at any time.
3. To block nonspecific background staining, the sections are completely covered with normal serum from the same species that provides the second antibody. For example, if the second antibody is to be goat anti-mouse, block with normal goat serum. The blocking serum is diluted with PBS antibody diluent (1 : 20). Incubate the sections at RT for at least 10 min.
4. Block endogenous biotin: Some tissues such as the liver and kidney have endogenous biotin and this must be blocked using the avidin–biotin kit from DAKO to avoid excessive background staining. Remove the blocking serum and cover the sections with avidin, leaving it for 15 min at RT.
5. Rinse the sections with PBS three times for 5 min each.
6. Cover sections with the solution 2 biotin and leave for another 15 min.
7. Rinse the sections with PBS three times for 5 min each.
8. Apply the primary (rabbit or mouse) antibody appropriately diluted with the PBS antibody diluent. Incubate for 2 h at RT or overnight at 4°C.
9. Rinse in PBS three times for 5 min each.

10. Apply the second antibody, biotinylated (swine anti-rabbit or goat anti-mouse) Ig depending on the primary antibody. This should be diluted appropriately (*see* **Note 3**). Leave for 30 min at RT.
11. Rinse in PBS three times for 5 min each.
12. Apply the third layer (peroxidase-labeled streptavidin) at the appropriate dilution (1 : 250 or 1 : 500) and leave for 30 min at RT.
13. Rinse in PBS three times for 5 min each.
14. Develop peroxidase. This step must be carried out in fume hood wearing a pair of gloves (*see* **Subheading 2.7.**). Take an aliquot of the DAB; if frozen, thaw it by placing the tube in a preheated water. For every 1 mL of DAB stock solution, use 100 mL of PBS and 33 µL of 30% hydrogen peroxide. The H_2O_2 is added last, just before immersing the slides. Leave slides in PBS/DAB/H_2O_2 solution for 10 min. Transfer slides to PBS and examine positive control slides (sections) under a light microscope. If the positive control has a dark brown staining in the appropriate location, place slides in running water. Also, examine a few test slides (sections) for the antibody; if they are all right, then proceed.
15. Counterstain lightly in Hematoxylin (about 30 s), rinse in running tap water, differentiate in acid alcohol, rinse in running tap water, blue in Scott's tap water, then back into the running tap water. (The Hematoxylin will stain the nuclei blue, and the acid alcohol will take off excess Hematoxylin from the staining). Check microscopically for light blue nuclei staining; if satisfied, proceed to dehydration.
16. Dehydrate the sections by placing them in changes of alcohol and xylene starting with 70% alcohol, through absolute alcohol, then xylene for about 2 min in each.
17. Mounting: Use DPX mountant, put the slides down with section facing upward, then put a drop of the mountant on the section, and cover with cover slips. Examine under a microscope.

4. Notes

1. Few antigens may be adversely affected by proteases and there is also a possibility that large protein molecules may be cleaved by the enzyme to smaller molecules, this may lead to a false-positive reaction for the peptide. Different protein should be digested for different times, likewise same antigen may require different times of digestion in different tissues.
2. When using the heat-mediated antigen retrieval, there is a possibility of the sections coming off the slides.
   a. Ensure that the slides are properly coated with poly-L-lysine, or use precoated polysine slides.
   b. The heat may be too high, so determine the optimum time that will be appropriate for each antigen.
3. To find the appropriate dilution for the antibodies, perform antibody titration for both the primary and second antibodies. The second antibodies are normally used at the dilution of either 1 : 250 or 1 : 500. Biotinylated swine anti-rabbit is normally used when the primary antibody is from rabbit, and for a mouse monoclonal primary antibody, the second antibody is biotinylated goat anti-mouse.

4. The most common cause of failure in day-by-day immunostaining is human error—taking the wrong bottle from the fridge, making an error in calculating a dilution, not checking a pH. Where both the control and test staining have failed and fault cannot be found along these lines, repeat the method, as before, with new preparations. Ensure that all reagents are labeled correctly.
5. If there is a high level of background staining, try the following:
   a. Dilute the primary antibody further.
   b. If this is ineffective, check the negative control with second (and third) reagents only. If background absent, the staining must be the result of a reaction between the primary and tissue that is detected by the second antibody ***(9)***. If background is still present it could be the result of the following:

I. Tissue factor (nonspecific binding sites)
   (a)Increase concentration of the blocking serum.
   (b) Add detergent to the rinsing buffer (e.g., 0.05% Tween-20).
   (c)Raise sodium chloride content of antibody diluent to 2.5%.

II. Endogenous biotin. Block endogenous biotin using the avidin–biotin kit

III. Incompletely blocked endogenous peroxidase
   (a)Try longer and stronger blocking with hydrogen peroxide; however, with frozen sections, hydrogen peroxide can damage some antigens, particularly a cell-surface antigen. Use a milder blocking method as described in **Subheading 3.2.**
   (b) Use another enzyme label instead of peroxidase.

IV. Cross-reaction between anti-species immunoglobulin (second antibody) and host tissue immunoglobulin.
   (a)Absorb crossreacting antibody with 1% of host tissue species normal serum or immunoglobulin.
   (b) Use species specific antibodies.

V. Aldehyde group in tissues left from fixative
   (a) Wash tissue well before processing and embedding.
   (b) Treat preparation with freshly made 0.02–1% sodium or potassium borohydride in 0.1*M* phosphate buffer or water for about 30 min at room temperature.
   (c)Add 10–100 mM ammonium chloride in the blocking serum.

6. Immunostaining weak or absent

I. Method sensitivity is insufficient for small quantity of antigen present. Increase sensitivity using the immunogold reaction with silver identification (*see* **ref. *9***).

II. Antigen is hidden due to fixation. Formalin and glutaraldehyde fixatives form crosslinks between the reactive groups of adjacent protein chains, making the antigens (proteins) inaccessible to the antibodies. Make sure you perform one of the following procedures, depending on the antigen type, to breakdown the hydroxyl bonds formed by the fixative with the protein (*see* **Subheading 3.3.**).
   (a) Antigen retrieval by protease treatment.

(b) Heat-mediated antigen retrieval.

III. Antibody deteriorating
   (a)Check all primary antibody against known positive controls with known positive second and third reagents.
   (b)Check second antibodies against primary known to be working.

IV. Wrong anitbody sequence applied in error
   (a)Rescue if possible by reapplication of antibodies.
   (b)Start all over again

V. Error in preparation of antibody, developing solution or any other reagent. Start again.

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9

Assessing Matrix Metalloproteinase Expression and Activity in Hepatocellular Carcinomas

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1. Introduction

The matrix metalloproteinases (MMPs) constitute a large family of zinc- and calcium-dependent endopeptidases that cleave extracellular matrix components ***(1)***. Hence, MMPs are classified according to their substrate specificities: interstitial collagenases, stromelysins, gelatinases, membrane-type matrix metalloproteinases (MT-MMPs), and elastase **(Table 1)**. The regulation of MMP activity involves gene expression, proteolytic processing of the propeptides to active forms, and inhibition by specific tissue inhibitors of matrix metalloproteinase (TIMPs). MMPs are involved in situations that require extracellular matrix remodeling, including wound healing, development, inflammation, fibrosis angiogenesis, and tumor invasion ***(2–5)***. Several complementary methods have provided an insightful description of the expression levels of MMPs and their pathological correlates. These include immunohistochemistry and Northern, Western, and dot blots. Additionally, the activity of MMPs is evaluated by gel substrate analysis. This approach has demonstrated that an increase in the expression of MMP2 ***(6,7)***, MT1-MMP ***(7)***, TIMP1, and TIMP2 ***(8–10)*** is associated with liver fibrosis. Similarly, in hepatocellular carcinomas, a high expression of MMP2, MMP9, MT1-MMP, and matrilysin is related to tumor aggressiveness ***(11–14)***. Consistently, by gel substrate analysis, MMP2 activity is increased in primary and secondary liver cancers ***(13,15,16)***. By *in situ* hybridization, the sources of MMP2, MT1-MMP, and TIMP2 are stromal cells, whereas the cellular origins of MT2-MMP are hepatocytes and bile duct cells ***(15,16)***. The study of MMP2

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

Table 1
MMP Family

Groups	Nomenclature		Substrates
Interstitial collagenases	MMP-1		Fibrillar collagens III>I, Type X collagen
	MMP-8	(PMN)	Fibrillar collagens I>III>II
	MMP-13	(collagenase 3)	Fibrillar collagens II>I,III, gelatin
Stromelysins	MMP-3	stromelysin 1	Laminin, fibronectin, nidogen, proteoglycans
	MMP-10	stromelysin 2	Laminin, fibronectin, nidogen, proteoglycans
	MMP-11	stromelysin 3	α1-Proteinase inhibitor
	MMP-7	matrilysin	Laminin, fibronectin, nidogen, proteoglycans
Gelatinases	MMP-2	gelatinase A	Collagens IV,V,VII,X,XI, gelatin, fibronectin, laminin, proteoglycans
	MMP-9	gelatinase B	Collagens IV, gelatin, fibronectin, laminin, nidogen, proteoglycans
Membrane-type MMPs	MMP-14	MT1-MMP	Fibrillar collagen, gelatin, fibronectin, laminin, nidogen, proteoglycans
	MMP-15	MT2-MMP	Fibronectin, laminin, nidogen, proteoglycans
	MMP-16	MT3-MMP	?
	MMP-17	MT4-MMP	?
Elastase	MMP-12		Elastin, fibronectin, laminin,nidogen, proteoglycans

activation in the liver relies on several tissue culture models. These include primary cultures and cocultures associating stromal cells and hepatocytes to study the role of cell–cell interactions in MMP2 activation ***(17)***. Thus, plastic-activated Hepatic Stellate Cells (HSC) in vitro secrete pro-MMP2, TIMP1, TIMP2, and MT1MMP ***(10,16,18,19)*** and Kupffer cells secrete MMP9 ***(20)***. However, in vitro activation of MMP2 requires interactions with hepatocytes, as has been demonstrated in cocultures of rat HSC and hepatocytes ***(17)***.

As a molecular pathology approach to the study of MMPs in hepatocellular carcinoma, the following sections describe the handling of liver samples to assess mRNA expression and MMP activity. Frozen biopsies enlarge the possibilities of purifying mRNA, nuclear factors ***(21)***, and functional MMPs ***(16)*** of high quality for research purposes. However, as these procedures neither result in direct benefit for the patient nor interfere in any way with the routine diagnosis by the anatomic pathology laboratory, the investigator must ensure that frozen samples are representative. Thus, we present biopsy bank management criteria useful in achieving sampling consistency and describe the optimization of quantitative dot blot and gel substrate analyses. In addition, characterization of MMPs in human liver cell populations by culture and *in situ* hybridization is presented. Ultimately, these methods should result in two-way technology transfers between basic research and reference pathology laboratories.

2. Materials

2.1. Management of Tissues Samples

Materials needed for tumor sampling and freezing: a dissecting board in high-density polyethylene, scalpel blades, cryogenic vials, cryogenic markers to label vials, one cryogenic plastic or glass beaker, two stainless-steel tweezers, long-handled wooden tweezers, isopentane, and a small portable container for liquid nitrogen.

2.2. Immunohistochemistry

1. Tissue sections: Freeze-dried tissue sections are obtained from approx $1 \times 1 \times 1$-cm frozen-tissue blocks. Mount tests and controls on the same slide and draw a hydrophobic circle around each of them with a hydrophobic marker (e.g., Dakopen; Dako, Glostrup, Denmark).
2. 10X phosphate-buffered saline (PBS): 30 m*M* $NaH_2PO_4 \cdot H_2O$, 70 m*M* $Na_2HPO_4 \cdot 2H_2O$, 1.3*M* NaCl, adjusted to pH 7.4 with 1*N* NaOH. Filter through 0.45 µm and autoclave. (*see* **Note 1.**)
3. Paraformaldehyde (PFA), 4% solution in 1X PBS (w/v). Weigh 20 g of PFA, add 50 mL of 10X PBS, and adjust to 450 mL with distilled water. Heat the milky suspension in a microwave oven under a fume hood until it becomes transparent. Avoid boiling. Let cool to room temperature and adjust to pH 7.4 with 1*N* NaOH

and to 500 mL with distilled water. Filter through 0.45 µm and keep up to 2 wk at room temperature. (*see* **Note 2.**)

4. H_2O_2, 0.3% solution in 1X PBS; prepared extemporaneously.
5. NH_4Cl, 50 m*M* solution in 1X PBS; prepared extemporaneously from 1M NH_4Cl.
6. Triton-X-100 (Sigma, St Louis, MO), 0.1% solution in 1X PBS; prepared extemporaneously.
7. Blocking solution: 2% bovine serum albumin (BSA) (Sigma) in 1X PBS.
8. Secondary antibody conjugated to horseradish peroxidase (e.g., blotting-grade affinity-purified goat anti-mouse or anti-rabbit IgG (H + L) (Biorad, Hercules, CA).
9. Substrate for horseradish peroxidase, prepared extemporaneously:
   a. Wear gloves and work under a fume hood. Weigh 4 mg of diaminobenzidine tetrahydrochoride (DAB) (Sigma) and add it to 10 mL of 50 mM Tris–HCl, pH 7.4.
   b. Add 100 µL of 30% H_2O_2 (Sigma) to 9.9 mL of sterile distilled water.
   c. Add 100 µL of item b to item a and vortex. The substrate is active for approx 30 min at room temperature. (*see* **Note 3.**)
10. Mounting medium: Kaiser's glycerol gelatin for microscopy (Merck). As its melting point is approx 40°C, heat the bottle in a microwave oven at 800 W for approx 10 s and keep it in a 40°C water bath during mounting.

2.3. In Situ Hybridization

1. In vitro transcription: ^{35}SrUTP (1250 Ci/mmol) (Amersham, Buckinghamshire, UK) and Riboprobe *In Vitro* Transcription kit (Promega, Madison, WI).
2. Fixation:
   - 4% PFA (*see* **Subheading 2.2.**).
   - Diethyl pyrocarbonate (DEPC)-treated water (*see* **Note 4**).
   - DEPC-treated 10X PBS.
   - 30%, 70%, and 90% ethanol dilutions in DEPC-treated water (v/v) and 100% ethanol.
3. Permeabilization of the tissue sections:
   - 0.2*N* HCl in DEPC-treated water.
   - Pronase (7000 U/g, Boehringer Mannheim, Meylan, France) 0.125 g/mL in 1X PBS. Prepare and activate it before use: Add 3.1 mL of DEPC-treated water to 125 mg of pronase. Incubate at 37°C for 4 h. Aliquot, lyophilize, and store at –20°C.
   - 0.1*M* glycine (Sigma) in 1X PBS. Add 0.1% DEPC; let stand at room temperature overnight and autoclave.
   - 0.1*M* triethanolamine (Sigma) in DEPC-treated water. Adjust pH to 8 with 1*N* HCl. Store at room temperature. Extemporaneously add 0.25% (v/v) acetic anhydride (Sigma). (*see* **Note 5.**)
4. Hybridization:
   - Air-tight plastic boxes where the slides will sit flat during hybridization, water bath set to 80°C, hybridization oven set to 50–52°C.
   - Deionized formamide. Add 30 g of analytic-grade Mixed Bed Resin AG-501-

X8 (D) (Biorad) to 500 mL of formamide (Merck) in an RNAse-free bottle. Let stand overnight at room temperature. Filter.

- 1*M* phosphate buffer: 3.5 g $NaH_2PO_4.H_2O$ and 4.3 g $Na_2HPO_4.2H_2O$, adjust volume to 50 mL with DEPC-treated water.
- RNAse-free 100X Denhardt's solution (Sigma).
- 10X hybridization buffer: 5*M* NaCl, 30 ml + 1*M* Tris.HCl pH 7.5, 5 mL + 1*M* phosphate buffer, 5 mL + 0.5*M* EDTA pH 8, 5 mL + RNAse-free 100X Denhardt's solution, 500 µL + 4.5 mL DEPC-treated water.
- 1*M* dithiothreitol (DTT).
- Yeast transfer RNA (ytRNA), 50 mg/mL in DEPC water.
- 50% dextran sulfate (w/v) in DEPC-treated water (*see* Note 6).
- Hybridization solution: 50% of deionized formamide, 12.5% of 10X hybridization buffer, 9% of DEPC-treated water, 1% of 1*M* DTT, 2.5% of ytRNA, 25 % of 50% dextran sulfate.
- 5X probe solution: The volume of the 5X probe solution will be one-fifth of the volume of the probe mixture applied to the tissue sections (*see* **Note 7**). Thus, the 5X probe mixture will contain 50% formamide, 10 m*M* DTT, DEPC-treated water, and 40×10^3 cpm/µL of ^{35}S-labeled probe.
- Probe mixture: Add one-fifth of 5X probe solution to four-fifths of hybrid ization solution, vortex vigorously, and spin briefly. The specific activity will thus be 8×10^3 cpm of ^{35}S-labeled probe /µL of probe mixture.

4. Posthybridization washings (*see* **Note 8**).
   - Rocking water bath set to 50–52°C.
   - Buffer 1: 50% formamide + 10% 10X hybridization buffer + 40% sterile distilled water + 0.15 % DTT (w/v). Buffer 1 should be heated to 50°C just before use.
   - Buffer 2: 100 m*M* Tris–HCl pH 8 + 1 m*M* EDTA pH 8 + 500 m*M* NaCl.
   - RNAse A from bovine pancreas, activity = 50 U/mg (Boehringer). Stock solution: dissolve 10 mg/mL in water, incubate for 2 min in a boiling water bath to inactivate endogenous DNAse activity. Store at –20°C in 1-mL aliquots. RNAse A, working solution: 20 µg/mL in buffer 2. Prepare and preheat to 37°C just before use.
   - 20X standard saline citrate (SSC), 2X SSC, and 0.1X SSC as described in molecular biology manuals ***(22)***.
   - 30%, 70%, and 90% ethanol dilutions in water and 100% ethanol.
5. Autoradiography:
   - Ilford G5 emulsion (Ilford, Lyon, France). The emulsion should be stored at 5°C, protected from light and radioactivity.
   - Darkroom. Check that the safelight conditions of your darkroom fit the requirements set by the manufacturer of the emulsion (*see* **Note 9**).
   - Dipping vessel (Amersham-Pharmacia Biotech, Les Ulis, France).
   - Heating water bath set to 42°C, under safelight conditions.
   - Racks to let emulsion-coated slides dry in vertical position.
   - Microscope slide storage boxes. These should be adapted to safelight condi

tions for exposure at 4°C. A convenient way is to wrap them with aluminum foil.
- $CaCl_2$ pellets (Merck) is used as a desiccant during exposure. Prepare paper pouches containing approx 10 g CaCl2 per 50-slide storage box.

6. Slide processing:
   - Kodak D-19 developer and Ilford Hypam fixative are available from local retailers. Prepare dilutions as recommended by the manufacturer.
   - Glacial acetic acid, 1% solution in tap water at approx 18°C.

2.4. Dot and Northern Blots

Reagents are described in routine molecular biology manuals (see, for example, **ref.** *22*).

2.5. Zymography

1. Sample buffer: 250 m*M* Tris–HCl pH 6.8, 10% sodium dodecyl sulfate (SDS), 4% sucrose, 0.05 % Bromophenol Blue.
2. Washing buffer: 2.5% Triton-X-100.
3. Reaction buffer: 50 m*M* Tris–HCl pH 7.4, containing 5 m*M* $CaCl_2$ and 1 μ*M* $ZnCl_2$.
4. Staining of zymograms: 30% methanol/10% acetic acid, containing 0.5% Coomassie Brilliant Blue G 250.

2.6. Cells and Media

Hepatocytes, hepatic stellate cells, Kuppfer cells, and endothelial cells are isolated from histologically normal livers. All reagents and culture media are described elsewhere ***(23-25)***.

3. Methods

3.1. Sampling of Hepatocellular Carcinomas and Controls

Access to the biopsy material must comply with national laws and with the requirements of the local and/or national ethics committee(s). Matching pairs of tumor and distant nontumor liver specimens are obtained from patients undergoing resection of hepatocellular carcinoma (HCC). Control samples are obtained from macroscopically normal partial hepatectomy specimens from patients undergoing resection of liver metastases or from liver donors excluded from the transplantation protocol because of extrahepatic disease unrelated to the cause of death. Sampling for experimental purposes is performed on the specimen at the anatomic pathology laboratory, immediately after resection and before its immersion in formalin. After macroscopic evaluation and sampling of the margins of surgical resection, the pathologist chooses the areas to be sampled following two criteria: (1) Sampling for research purposes must not interfere with the routine assessment of the specimen for clinical purposes; (2) the information gathered from frozen material (e.g., tumor heterogeneity,

capsule, capsular invasion) must be consistent with that obtained from the material fixed in formalin and embedded in paraffin. All paraffin-embedded specimens are routinely processed for histology (i.e., Hematoxylin–Eosin–saffran and Sirius Red staining) and examined by routine pathologists unaware of the molecular pathology data for each case. For research purposes, a part of the fresh material is snap-frozen in isopentane cooled in liquid nitrogen and stored at –80°C until use.

1. Sample the specimen chosen by the pathologist. Using gloves and protective goggles, prepare approx 1×1×1-cm cubes with a No. 24 scalpel blade (*see* **Note 10**).
2. Separate the groups of samples (i.e., tumor, nontumor area), label the cryogenic vials accordingly, and let them stand in liquid nitrogen. Pour approx 50 mL isopentane in a 100-mL beaker, take it with the wooden tweezers, and partially immerse it in liquid nitrogen (*see* **Note 11**).
3. When an adequate temperature has been reached, immerse the tissue blocks *one by one* in cold isopentane and let them snap-freeze for approx 30 s (*see* **Note 12**). Insert the blocks in the corresponding vial and keep them in the liquid-nitrogen container until sampling has been completed (*see* **Note 13**).

3.2. Biopsy Bank Management

3.2.1. Long-Term Storage

The complexity and the security levels required vary according to the number, the turnover, and the biohazard features of the samples stored. The minimal requirement for long-term storage at –80°C is an ultralow-temperature freezer. Its specifications include two (1000-W) compressors, proximity alarms (buzzer and lamp), remote alarm, and CO_2–injection security system.

3.2.2. Management of Clinical, Pathological, and Sampling Data

Several computer programs are available that allow storage and retrieval of demographic, biological, diagnostic, and therapeutic data for each patient and creation of links with sample management data.

3.2.3. Ensuring Sampling Consistency in a Biopsy Bank

Several controls are required downstream in the processing of tissue samples:

1. In normal livers, the absence of significant histological lesion is checked before the inclusion of these samples as controls.
2. In hepatocellular carcinomas, only those frozen-tissue samples with anatomic pathology features that allow a matching diagnosis with the pathology report of each patient are homogenized for RNA or protein extraction.
3. The size of the frozen blocks has been optimized to at least 1 cm^3 in volume for large tumors; thus, sampling large areas ensures that clonal heterogeneity in HCC is not a confounding variable.

4. When heterogeneity is macroscopically obvious (e.g., nodule-in-nodules, areas different in aspect, color, or density), the different areas are separately labeled and stored.
5. Necrotic or congestive areas are excluded; this ensures consistency in RNA yield. Samples that do not comply with these criteria at any stage of their processing are either reassigned to the right category or discarded.

3.3. MMP Expression

3.3.1. Immunohistochemistry

Immunohistochemistry allows the localization of MMP and TIMP proteins. We use it to characterize the cells expressing MMP2, MT1MMP, MT2MMP, or TIMP2 mRNA, as detected by *in situ* hybridization (*see* **Subheading 3.3.2.**). Immunodetection of stromal cells with features of smooth muscle or vascular differentiation is performed with anti-α-SM-1 antibodies, recognizing α-smooth muscle actin (αSMA) (Sigma) or anti-von Willebrand factor (vWF) (DAKO) antibodies, respectively. The distribution of αSMA or vWF by immunohistology and MMP2 and TIMP2 transcripts by *in situ* hybridization are compared on contiguous serial sections ***(15,16)***. All incubations are done at room temperature.

1. Five-micrometer-thick frozen sections are fixed in 4% paraformaldehyde at room temperature for 15 min, rinsed three times for 1 min and once for 5 min in PBS and subsequently incubated in 0.3% H_2O_2, 50 m*M* NH_4Cl, and 0.1% Triton-X-100 for 15 min each. (*see* **Note 14**.)
2. After washing in PBS, sections are incubated in blocking solution for 1 h.
3. Primary antibody is applied in blocking solution at room temperature for 60 min.
4. After rinsing in PBS, sections are incubated with peroxidase-conjugated affinity-purified antibodies against the species in which the primary antibody has been raised.
5. Rinse in PBS and apply the peroxidase substrate for 20 min. Rinse in PBS, counterstain with Hematoxylin and mount with glycerol gelatin.

3.3.2. In Situ *Hybridization*

1. Linear expression vectors carrying the appropriate inserts are used for in vitro transcription in the presence of 60 mCi ^{35}S rUTP (*see* **Note 15**).
2. Five-micrometer frozen sections mounted on slides are fixed in 4% paraformaldehyde at room temperature for 20 min, rinsed once for 5 min in 3X PBS and in 1X PBS, respectively and dehydrated in subsequent bathes with 30%, 70%, 90%, and 100% ethanol, for less than 2 min each. Then, they are let to air-dry at room temperature for approx 1 h. (*see* **Note 16.**)
3. Permeabilization: All incubations are performed at room temperature. Incubate the slides in 0.2*N* HCl for 20 min, rinse them in DEPC-treated water once for 5 min, then incubate them in the pronase solution for 10 min and in 0.1*M* glycine for 30 s, postfix them in 4% PFA for 20 min and rinse three times for 1 min in 1X PBS. While the slides are in the last PBS bath, add 0.25% acetic anhydride (v/v) to the

0.1*M* triethanolamine solution and shake vigorously, then incubate in this solution for 10 min, rinse once for 5 min in 1X PBS, and dehydrate through graded ethanol solutions.

4. Hybridization is carried out overnight at 50–52°C with probe mixture (*see* **Note 17**).
5. Perform two stringency washings with buffer 1 at 50°C. The first for 1 h and the second for 4 h (*see* **Note 18**.)
6. Equilibrate the slides in buffer 2, incubate in RNAse A working solution, and again in buffer 2 at 37°C for 30 min each. Place the racks with the slides in dishes containing 2X SSC at room temperature. Incubate in 2X SSC and in 0.1X SSC under gentle rocking for 30 min, respectively. Then, dehydrate through graded ethanol solutions (*see* **Note 19**). Let air-dry.
7. Coat the slides with photographic emulsion. Melt the emulsion in a 42°C water bath in complete darkness for 2 h. Pour it into the dipping vessel and dilute with distilled water. Stir gently with a blank slide and let homogenize for 15 min. Check the thickness of the emulsion and adjust it as required. Gently dip the slides in the emulsion, one by one, for 2–3 s. Let them dry for at least 2 h in a humid atmosphere. Then place them in microscope storage boxes. Expose at 4°C for the required time. (*see* **Note 20**.)
8. Process the microautoradiographies following the guidelines of the manufacturer of the emulsion. Let the slide storage boxes equilibrate to room temperature a few hours before processing. In the darkroom, incubate in the developer solution for 3 min, quench in 1% acetic acid for 30 s and then incubate in the fixative for 6 min. Rinse with running tap water for 10 min and stain with Hematoxylin and Eosin. (*see* **Note 21**.)

3.3.3. Methods for Quantification of mRNA

Several methods are available to analyze mRNA levels of MMPs, their inhibitors and extracellular matrix components. They include Northern blots, quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) ***(26)*** and dot blots ***(16)***. Both RT-PCR and dot blots allow rapid and reproducible quantitative results. RT-PCR enables one to carry out mRNA analysis in archival paraffin-embedded material ***(27)***, but for each one of the molecular markers tested, it requires the optimized design of primer sets, vectors for competitive amplification or amplification of ubiquitous sequences to normalize mRNA degradation, and, eventually, quantitative analysis of the RT-PCR products by dot blot, capillary electrophoresis ***(28)***, or Southern blots ***(26)***. Two recently described methods include *cDNA microarrays* ***(29,30)***, whereby the expression levels of thousand of genes within one tumor can be determined in a single experiment and *tissue microarrays* ***(31)***, constituted of small-core biopsies punched from microscopically selected regions in archival paraffin blocks. The latter allow the comparative analysis of mRNA or protein of several hundreds of tumors at a time by *in situ* hybridization or imunohistochemistry.

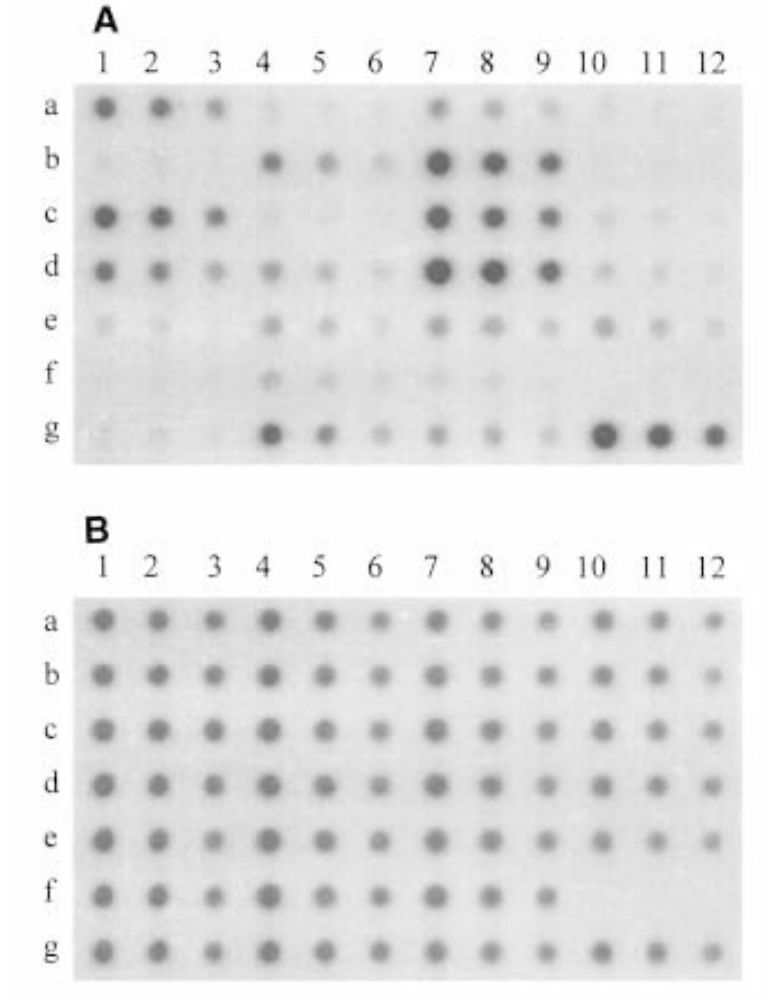

Fig. 1. An example of quantitative dot-blot analysis for MMP2 (A) and 18S ribosomal RNA (B) in human HCCs (**Subheading 3.3.3.1.**). Total RNA from hepatocellular carcinomas ($n = 27$) was blotted in triplicate at 5, 2.5, and 1.25 µg/µL onto a Hybond N^+ nylon membrane using a filtration manifold. The respective serial dilutions were blotted from positions a1, a2, and a3, for sample 1, to positions g10, g11, and g12, for sample 28. Positions f10, f11, and f12 (sample 27) are occupied with serial dilutions of yeast transfer RNA as a negative control. Total RNA samples were blotted in 200 µL of 4X SSC, 4 mol/L formaldehyde, 0.3 mol/L NaAc, and 0.002% Methylene Blue. Membranes were dried on Whatmann 3M paper at 65°C for 10 min and ultraviolet crosslinked. Blotting efficiency was assessed by Methylene Blue staining. The MMP2 cDNA probe ***(16)*** was labeled to high specific activity (1×10^8 to 1×10^9 cpm/µg

3.3.3.1. Quantitative Dot Blots

Dot blots are mRNA arrays that enable simultaneous analysis of a large number of samples in one experiment, provided high-quality mRNA is available from frozen samples **(Fig. 1)**. Thus, quantitative comparisons of mRNA levels are performed among different liver diseases, under the same experimental conditions ***(16,21)***. In addition, nylon membranes used for dot blots can be stripped and reprobed, which considerably increases the possibility of testing the same group of samples for several markers. Relative mRNA amounts are corrected for minor differences in RNA loading by normalization with the signal obtained for 18S rRNA. Typical experiments use membranes with 96 points each (up to 8 membranes per experiment). ^{32}P-labeled cDNA probes may be used and autoradiography films are exposed to the membranes. Automated densitometry readings of autoradiographs are performed using commercially available software. The results thus obtained are readily copied–pasted onto the usual data analysis software. Although the protocol for dot blot is essentially that described for Northern blots, the success of the analysis depends on linear-range mRNA detection. In addition, because densitometry readings are automated, a perfectly "clean" and homogenous background must be obtained.

1. Before starting a dot-blot experiment on a series of mRNA samples, optimize mRNA detection to ensure that it is performed within the linear range. This may be done with in vitro-transcribed mRNA for the probes of interest. It is suggested that at least five different standard dilutions be used. Alternatively, perform five dilutions of total mRNA from a sample already known to contain high RNA levels for the probe of interest. (*see* **Note 22.**)
2. The protocol for RNA loading and detection by dot blots is described in standard molecular biology manuals ***(22)***. Positively charged nylon membranes can be stripped and reprobed up to six times, provided special care is taken to avoid irreversible binding of the cDNA probe (*see* **Note 23**).

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cDNA) using the Rediprime kit (Amersham) and 3000 Ci/mmol α-dCTP ^{32}P (Amersham). Hybridization was performed in 0.5 mol/L NaPi containing 7% SDS, 1 mmol/L EDTA, and 1% BSA for 16 h at 65°C. Filters were washed in 3X SSC/0.1% SDS three times for 1 min and twice for 15 min at 65°C, followed by 0.5X SSC/0.1%SDS twice for 15 min at 65°C. Films were exposed with enhancing screens at –80°C. For each probe, all samples were run in the same experiment and exposed simultaneously to the same film. Hybridizations were performed under probe concentrations and film exposure times that yielded a linear relationship between densitometry signal and amount of RNA loaded (range tested, 1–5 µg RNA; $r = 0.97–0.99$). To correct for differences in the amount of RNA in each dot, hybridization with a 25-mer oligoprobe for 18S ribosomal RNA (B) was performed. Using the densitometry readings obtained from B, each densitometry signal was normalized to reflect equivalent total RNA in each blot. Values are thus expressed as MMP2/18S ratios.

3.3.4. Liver Cell Cultures

Liver cells are obtained by dissociation of histologically normal liver fragments. Detailed procedures are described for human hepatocytes ***(23,32)*** and sinusoidal cells ***(24)***. Human hepatic stellate cells can be used until passage 10.

3.3.5. MMP Activity

Zymography analysis is a convenient methodology ***(33)*** to characterize the nature of MMPs and the amount of both the proform and the active enzyme.

1. Samples: Seven-micrometer-thick frozen sections making up a surface of 1 cm2 are scraped off the slides with scalpel blades and homogeneized in 50 µL of sample buffer. For cell cultures, vortex 15 µL of conditioned medium with 5 µL of sample buffer and load (*see* **Note 24**).
2. Electrophoresis is performed in SDS–7% polyacrylamide gels copolymerized with 1 mg/mL gelatin (substrate of MMP2 and MMP9) or casein (substrate of stromelysin) (*see* **Note 25**).
3. After migration, gels are rinsed twice at room temperature for 10 min in 2.5% Triton-X-100 to wash off SDS and twice for 10 min in water to remove Triton-X-100.
4. Incubate the gel in reaction buffer at 37 °C, overnight (*see* **Note 26**).
5. The gel is colored in Coomassie Brilliant Blue G250 and contrasted in 30% methanol/10% acetic acid/60% distilled water. MMP activity appears as clear lysis bands. The colored gels may be thus stored in plastic bags at 4°C for several weeks. The amount of the pro-enzymes and the active enzymes are analyzed by densitometric scanning. To perform quantitative comparisons between different gels, include a standard in each gel (*see* **Note 27**).

4. Notes

1. Milli-Q water or equivalent water with >18.2 MΩ resistivity should be used to prepare all the reagents described in this chapter, unless otherwise specified.
2. When working with PFA, always wear gloves, goggles, and work under a fume hood. PFA may cause sensitization by skin contact, respiratory troubles, and nasal bleeding from inhaled vapors.
3. Refer to risk and safety data from the manufacturer. DAB is toxic by inhalation and by contact with skin and is a possible carcinogen. By contact with water, it liberates toxic gas. Toxic effects may be cumulative. Ready-to-use DAB peroxidase tablets are available from several manufacturers. The same risk and safety precautions apply to the tablets.
4. DEPC hydrolyzes in the presence of water to ethanol and CO_2, the latter causing internal pressure in the bottle. Thus, it must be stored at 4°C. DEPC is toxic by inhalation and in contact with skin. Always manipulate it under a fume hood. DEPC is suspected to be a carcinogen. Add 1 mL of DEPC (Sigma) to l L of distilled water. DEPC falls to the bottom of the bottle and does not dissolve spontaneously. Shake the bottle vigorously to help dissolution and let it stand at room temperature overnight. Then, autoclave for 15 min at 15 psi on a liquid

cycle. This treatment removes traces of DEPC that might modify purine residues in RNA by carboxymethylation ***(22)***. Most buffers and solutions may be treated with DEPC before being autoclaved. As DEPC reacts rapidly with amines, it cannot be used to treat Tris buffers. Prepare Tris buffers with DEPC-treated water once it has been autoclaved.

5. Acetic anhydride is active only for a short time after addition to 0.1*M* triethanolamine.
6. 50% Dextran sulfate should be prepared in advance and stored at –20°C.
7. Approximately, 25 µL of probe mixture is required for one 1-cm^2 tissue section.
8. DEPC-treated water is not necessary for posthybridization washings.
9. The temperature of the darkroom should be approx 25°C and 75% ambient humidity, as a cold dry atmosphere will cause the emulsion to crackle. As coating a large number of slides requires several hours' work; working in total darkness diminishes the background signal.
10. Liver samples should be considered biologically hazardous. Although subject to epidemilogical variations, HCC is frequently associated with hepatitis B and C virus infections.
11. We use commercially available polypropylene cryogenic vials with high-density polyethylene closure for long-term storage. The screw closures consist of internal threads that fit the external threads on the vial neck to ensure a tight closure. Usage must strictly conform to the manufacturer's guidelines to avoid explosion or biohazard release.
12. The adequate temperature is reached *just before* a few white, solid pill-like deposits form at the bottom of the beaker. Test the temperature with a tissue block. If it splits or cracks, it means that isopentane is too cold.
13. Samples should be snap-frozen within approx 15 min of resection to avoid endogenous RNAse- and protease-mediated damage. Although RNA and protein can be retrieved from biopsies standing for several hours at room temperature, the delay between resection and freezing should be standardized.
14. Triton-X-100 is a nonionic surfactant that solubilizes membrane proteins under nondenaturing conditions. The need for permeabilizeation depends on the epitope detected.
15. Schedule in vitro transcription for the day of delivery of ^{35}S rUTP. In vitro transcription performed with "fresh" ^{35}S rUTP will yield enough probe of high specific activity to allow hybridization of hundreds of sections. The half-life of ^{35}S rUTP is 87 d, so try to perform all hybridization within the first 3 wk of the date of calibration of the nucleotide to achieve high signal-to-noise ratios. This takes into account exposure time, which may reach 4 wk. The labeled probe may be stored at –80°C in 50% formamide. Before taking an aliquot, incubate at 65°C for 30 s and vortex.
16. Prehybridization and hybridization must be performed under RNAse-free conditions. Consider as contaminated all laboratory glassware that has not been treated to inactivate RNAses. Use a minimum of single-use plasticware and inactivate RNAses in glassware. Systematically inactivate RNAses in glassware after each use. Several methods are available to inactivate RNAses ***(22)***. We bake

glassware at 180–200°C for at least 8 h. Glass staining jars and dishes and other anatomic pathology glassware do not withstand rapid temperature variations and become brittle at high temperatures.

17. Before applying the probe, cut approx 1.5×1.5-cm^2 pieces of Parafilm™ (American National Can, Neenah, WI) and prepare the hybridization solution, the 5X probe solution, and the probe mixture(s); set aside flat-bottomed plastic boxes with close-fitting lids to incubate the slides. Be sure that the plastic boxes will not warp or open at 50°C. To apply the probe, complete the following steps under a fumehood and wear goggles: Dispense an aliquot over one of the corners of the section. Do not attempt to spread it onto the section as this may damage tissue structure. Gently apply a square of Paralfilm: Just allow tangential contact of the film with the drop and let the film roll down smoothly as it spreads the solution over the sections. Do not apply the film on top of the drop, as it will form an air bubble. The corners of the film may warp upward. They will spread out flat during incubation. Close the box.
18. The purpose of this step is to remove excess probe and to denature unspecific RNA–RNA bonds. Do not pull the films off the slides, as this damages the tissue sections. Preheat buffer 1 at 50°C in rectangular glass dishes with removable racks. Use a heating bath with gentle rocking (i.e., approx 40–60 oscilations/min). Immerse the slides and incubate for 1 hr. The films will come to the surface. Remove the floating films, change the washing buffer and incubate under agitation for 4 h. These films and the washing buffer are ^{35}S-contaminated waste. Dispose of accordingly.
19. After the 5-h stringency washings, specific RNA–RNA hybrids remain double stranded. In contrast, unspecific RNA–RNA bonds are denatured. Therefore, the unbound RNA probe remains single stranded. Whereas double-stranded RNA–RNA hybrids are not sensitive to RNAse A, single-stranded RNA is rapidly degraded into short oligonucleotides. Further washings in 2X and 0.1X SSC will remove them. From this step onward, do not work in the RNAse-free area of your laboratory.
20. Dilution in distilled water optimizes the thickness of the photographic emulsion. We prepare the working dilution as one part emulsion and two to three parts of distilled water. Drying after coating should be as gradual as possible, as harsh drying may result in emulsion crackling.
21. To process the autoradiographies, set the slides in vertical staining jars (with internal grooves and wide mouth). This facilitates pouring developer and fixative in the dark without slide damage. Staining with Hematoxylin and Eosin is performed under standard conditions. However, omit the acid–ethanol step after Hematoxylin staining, as acid treatment may result in loss of signal.
22. Densitometric data from autoradiography must bear a linear relationship with the amount of RNA loaded. Thus, the variables involved in the end result include abundance of the particular mRNA, cDNA probe concentration, specific activity of probe, stringency washings, exposure time, and film processing. Additionally, the settings of parameters such as "background correction" and "contrast" by the image analysis software may significantly affect the linearity of the final readings.

23. After stringency washings, pour abundant 0.5 SSC/0.1 % SDS buffer on a glass tray and immerge the membranes in it. Prepare two polyethylene sheets to the dimensions of the membranes. Set the membranes flat one by one on one polyethylene sheet and pour approx 5 mL of buffer on each. Apply the second polyethylene sheet and, with a paper pad or sponge, smooth out the bubbles or excess liquid gently. Do not let the membranes dry once they have been hybridized. Do not let the membranes sit over Whatmann paper after washings, as this may cause irreversible binding of the cDNA probe to the membrane.
24. The activation of MMP2 is a localized process that depends on the pericellular microenvironment ***(34)***. Thus, before scraping off the tumor section, examine contiguous sections by Hematoxylin staining. If the tumor section contains adjacent nontumor liver, dissect it out. To perform zymograms on conditioned medium from cultured cells, rinse and culture cells in serum-free medium for 24 h before sampling. This avoids background contamination of MMPs or inhibitors from fetal calf serum. Conditioned media are centrifuged to discard debris, and aliquots are immediately analyzed or stored at –80°C
25. Concentrated gelatin or casein may be stored in aliquots at –20°C.
26. Reaction buffer may be stored at 4°C up to 1 mo.
27. The active sites in MMPs, which are protected by the pro-peptides in the pro-enzymes, are exposed by the conformational change induced by SDS. This enables the detection of the latent MMPs by zymography.

Acknowledgments

We thank Dr. Bruno Turlin (Laboratoire d'Anatomie Pathologique, Hôpital Pontchaillou, Rennes, France) for sampling the surgical specimens and Dr. Jean-Pierre Campion, Dr. Karim Boudjema, and Dr. Dominique Lotrian (Service de Chirurgie Digestive et Transplantation) for collection of clinical data. This work was supported by the Institut National de la Santé et de la Recherche Médicale, the Secrétariat d'Etat à la Santé (Programme Hospitalier de Recherche Clinique 1997, DRRC Bretagne), and the Association pour la Recherche contre le Cancer.

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10

Isolation of Novel Markers for Hepatocellular Carcinoma by a Subtraction-Enhanced Display Technique

Chuan-Ging Wu

1. Introduction

With an estimated annual incidence of between 250,000 and 1 million cases worldwide, hepatocellular carcinoma (HCC) is one of the most frequent malignancies in humans and occurs mainly in areas of Asia and Africa *(1,2)*. Early diagnosis is critical for patients with HCC, because the 5-yr survival rate is significantly improved for patients with small, subclinical HCC after surgical resection *(3)*. Therefore, identification of gene products exclusively or abundantly expressed in HCC tissue may yield novel tumor markers that are sensitive and specific to HCC. Moreover, it could lead to the early detection and further efficient targeting of tumor, and eventually to cure the disease.

It is widely acknowledged that the development of malignancies results from a stepwise process involving multiple genetic events like activation of proto-oncogenes, inactivation of tumor-suppressor genes, and overexpression or re-expression of growth factors involving stages of initiation, promotion, and progression *(4,5)*. A variety of epidemiological, cytogenetic, and experimental studies have well delineated that tumors are caused by an accumulation of genetic damage *(6,7)*. Although detailed knowledge of the sequence and in vivo mechanistic effects of these alterations is rudimentary for most, if not all, tumors, their identification should, in principle, serve as ideal markers for the detection and monitoring of malignancies *(8)*. At a clinical level, these genes and their products may provide more precise diagnostic, prognostic, and even therapeutic characterization of individual tumors. This can be carried out at three levels, namely at DNA,

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

mRNA, and protein levels using fluorescence *in situ* hybridization (FISH), Southern blot, polymerase chain reaction (PCR), Northern blot, *in situ* hybridization, and monoclonal antibody assays against oncoproteins as markers with altered expression in cancer patients ***(9,10)***.

Despite a general acknowledgment that the majority of HCC cases are associated with interactive effects of viral and chemical carcinogens, the latter have been widely used for analyzing multistep carcinogenesis and for generating an animal model ***(11)***. Being a potent carcinogen, diethylnitrosamine (DENA) is considered to be a genotoxic tumor initiator. When administered continuously to rats, it produces well-characterized tumors in a dose-response relationship ***(12)***.

This chapter focuses on the description of a feasible procedure called subtraction-enhanced display, the combined technique of subtractive hybridization and differential display, whereby the upregulated and downregulated gene products were identified using rat HCC as a model. The main principle of the approach is based on the depletion of common gene products in both tumor and normal liver cells, thereby enriching the specific species as described by Wang et al. ***(13)***, and subsequently comparing and identifying the remaining upregulated or downregulated cDNA fragments on a display gel. The interesting cDNA fragments were directly subjected to full-length cDNA cloning, Northern blot analysis, *in situ* hybridization, and expression of protein if secreted, to raise antibody and so forth. These gene products could be potential useful markers for tumor detection and gene therapy.

2. Materials

2.1. Liver Specimens

Liver samples were from Wistar rats that had been chemically induced by supplying DENA (Sigma, St. Louis, MO) via the drinking water at a concentration of 1 : 10,000 for up to 4 mo ***(14)***. Tissue was confirmed as HCC by a standard histopathologic technique employing Hematoxylin & Eosin staining. Normal rat liver tissue was from the pair-fed controlled Wistar rats. All liver tissues were collected and stored at –80°C.

2.2. Other Reagents

1. TE buffer: 10 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.4.
2. 1X standard saline citrate (SSC): 0.15*M* NaCl/0.015 *M* sodium citrate.
3. 50X Denhardt's solution: 1% (w/v) Ficoll (Type 400, Pharmacia), 1% (w/v) polyvinylpyrrolidone and 1% (w/v) bovine serum albumin (Fraction V, Sigma) in H_2O.
4. Prehybridization solution: 6X SSC, 5X Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 100 µg/mL of herring sperm DNA.
5. Oligo(dT) cellulose (Boehringer, Almere, The Netherlands).
6. Oligo(dT)-Not I: disolved in TE (100 µg/mL) (Invitrogen, San Diego, CA).

7. Adapter 1: 5'CTCTTGCTTGAATTCGGACTA.
8. Adapter 2: 5'ACTTAGCAAAGGCTTGCC. (*see* **Note 4** for test tips.)
9. Super Taq DNA polymerase (Sphaero Q, Leiden, The Netherlands).
10. QIAquick-spin PCR purification kit: QIAGEN, Hilden, Germany.
11. Photo-biotinylation: 100 µg cDNA/100 µL photo-biotin (µg/µL) (Vector Laboratories, Burlingame, CA).
12. Streptavidine: 100 µg/mL (Gibco-BRL, Breda, The Netherlands).

3. Methods

3.1. Preparation of RNA and cDNA Fragments

1. Total cellular RNA is extracted from frozen liver tissue of both HCC and normal rats by a guanidium–thiocyanate–phenol–chloroform method ***(15)***.
2. Poly(A)$^+$ RNA is purified by means of oligo(dT) cellulose affinity chromatography.
3. Oligo(dT)-Not I (1.25 µg) is used to prime the first strand of cDNA synthesis from 2.5 µg poly(A)$^+$ RNA. (*see* **Note 2** for preparation tips.)
4. Double-stranded cDNA is synthesized using a Promega kit according to the manufacturer's instruction (Leiden, The Netherlands).
5. To prepare cDNA fragments for the subtraction procedure, the method as described by Wang and Brown ***(13)*** is used with minor modifications. In brief, cDNAs are digested with Alu I and Alu I/Rsa I for 1 h at 37°C. (*see* **Note 3** for preparation tips.)
6. The cDNA fragment populations derived from HCC and normal liver are ligated with adapters 1 and 2.
7. The ligation reactions are fractionated by electrophoresis through a 1.0% low-melting agarose gel in order to remove the unligated adapters.
8. The adapter-linked cDNA fragments in the size range of 0.1–1 kb are combined.
9. Each cDNA fragment population is amplified by PCR, utilizing Super Taq DNA polymerase and the respective specific primers. Amplification conditions are 94°C for 1 min, 53°C for 1.5 min, 72°C for 2 min, for 30 cycles, followed by a final elongation step of 5 min at 72°C.
10. The amplified products are purified with QIAquick-spin PCR purification kit.

3.2. Subtractive Hybridization

1. Subtractive hybridization is performed to deplete common gene products existing in both tumor and normal liver tissue. To monitor the feasibility of the procedure, a control experiment is paralleled throughout the whole experiment. The normal cDNA fragments are used as driver and the HCC cDNA fragments as tracer. For the control experiment, normal cDNA plus 0.2% phage ϕX174/*Hae*III DNA is used as a tracer (*see* **Note 1**).
2. cDNA from the driver cDNA population is amplified by PCR and photo-biotinylated by exposure to a 300-W sun lamp (Philips, The Netherlands) for 30 min.
3. Extract biotinylated cDNA fragments with an equal volume of 2-butanal three times.
4. In order to obtain the subtraction-enriched cDNAs, a 20-fold excess of the biotinylated driver cDNA is hybridized with 2.5 µg of tracer cDNA at 65°C for 20 h

(long cycle of hybridization, *see* **Note 5**).

5. After incubation with 100 µg of streptavidine for 30 min at room temperature, the biotinylated cDNA–protein complex is extracted with phenol/chloroform and followed by ethanol precipitation (*see* **Note 5**).
6. For the short cycle of hybridization, the above procedure is repeated for only 2 h of hybridization.
7. After the amplification by PCR (employing the specific primers) and purification, an additional round of long and short subtractive hybridization cycles are performed as described **(Fig. 1)**. (*see* **Note 6**.)

3.3. Display of Subtraction-Enriched cDNA Population

To analyze and isolate the subtraction-enriched cDNA fragments from both HCC and normal control liver, the principle of differential display technique ***(16)*** is combined. The enriched cDNA fragments obtained after subtractive hybridization, as well as the initial cDNA fragments derived from HCC and the control, are amplified using Taq DNA polymerase and the specific primers in the presence of 0.5 µ*M* [α-^{35}S] dATP (1200 Ci/mmol, final volume 25 µL). The experimental conditions are exactly the same as those used to generate the large quantities of cDNA needed for the actual subtractive hybridization, except that the concentration of nonradioactive dATP is lowered from 200 to 20 m*M* to obtain a sufficiently high specific activity. Comparable amounts of amplified radiolabeled cDNAs are then subjected to electrophoresis on a 6% polyacrylamide DNA-sequencing gel. An X-ray film (Kodak) is exposed to the dried gel overnight. (*see* **Note 7** for preparation tips.)

3.4. Cloning and Sequencing Procedure

1. Distinct cDNA bands of over 150 bases in length are individually excised from the display gel, and eluted in 100 µL of TE buffer.
2. 25 µL of the aqueous cDNA extract is amplified for 20 cycles by PCR with the specific primer.
3. The amplified cDNA fragments are directly cloned into the pCR™ vector (Invitrogen).
4. The recombined plasmid is transformed into INVαF' cells and plated out on ampicillin–IPTG (isopropylthio-β-D-galactoside)-X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)–agar plates.
5. Six white colonies from each plate are randomly isolated and analyzed for inserts on 1.2% agarose gel. Clones with inserts that varied in molecular size proceeded to the sequencing step.
6. Sequences of more than 150 bases are compared with those nucleotide sequences previously reported in the GenBank database, using the Blast search.

3.5. Northern Blot Analysis

1. Total RNA (20 µg) from HCC and normal liver tissue is separated on a 1% formaldehyde–agarose gel and transferred to Hybond-N nylon membrane (Amersham UK).

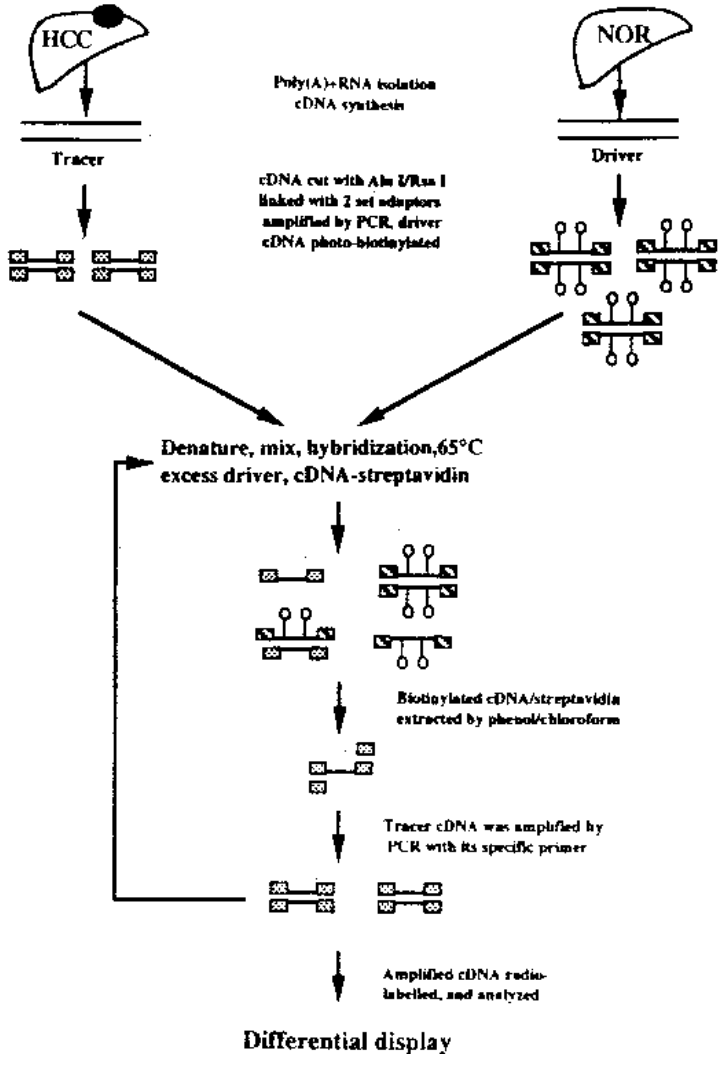

Fig. 1. A schematic representation of flow diagram is shown for the step of RNA isolation, cDNA synthesis, cDNA biotinylation, subtractive hybridization, PCR amplification, and purification. The cDNA derived from HCC liver as tracer was subtracted by driver cDNA from normal liver (Nor).

2. After fixation at 80°C for 2 h, the Northern blots are prehybridized for 2 h at 65°C in prehybridization solution.
3. These newly identified cDNAs are used as probes that are isolated from low-melting gel and labeled according to the hexamer-random-primed method following the vendor's protocol (Promega).

4. Blots are hybridized under the same conditions as stated for prehybridization.
5. The filters are washed four times for 15 min with 1X SSC/0.1% SDS and once with 0.2X SSC/0.1% SDS at 65°C.
6. Blots are exposed for Phosphorimager analysis.
7. The radiolabeled probes were then stripped from the membranes in a 0.1% SDS solution following manufacturer's protocol (Amersham) and rehybridized with ^{28}S rRNA probe.
8. The membranes are scanned with a Phosphorimager radioanalytic scanning system (Molecular Dynamics, USA) in order to quantify the amount of radioactivity of individual bands.

3.6. Detection of Subtraction-Enriched cDNA

In order to analyze the enriched cDNA fragments directly, cDNA populations prepared from HCC and the control are compared on a sequence gel before and after subtractive hybridization. In the control experiment, all phage DNA fragments showed up after subtraction, whereas they are hardly seen in the initial unsubtracted cDNA population (**Fig. 2**, right panel). This indicates feasibility and reliability of the technique and suggests the true enrichment of HCC-specific cDNA fragments in the experimental panel (**Fig. 2**, left panel). Furthermore, a few bands, as indicated with an arrowhead (**Fig. 2**, lane 4), abundant in the initial cDNA, disappear after subtraction, indicating that common cDNAs have been eliminated.

3.7. Analysis of Differential Expression in HCC and Normal Liver

In order to demonstrate the truly altered expression of these isolated gene products, the subtraction-enriched cDNA fragments are radiolabeled as probes to assess the abundance of the corresponding mRNA in 20 µg of total RNA from HCC and normal liver on Northern blot. As a paradigm of most known tumor-associated genes, ferritin-H is found to be more than 10-fold abundant in HCC as compared to that in normal liver tissue **(Fig. 3)**. Furthermore, many of these cDNAs can be directly applied as probes to detect the localization of their targeting mRNA by the *in situ* hybridization technique (data not shown).

4. Notes

1. Subtractive hybridization is a useful procedure to enrich those mRNAs (in the form of cDNA) that differ in concentration between two cell populations (i.e., tumor versus normal). The method is based on the depletion of common gene products, thereby enriching the specific species ***(13)***. There was need to minimize analysis of the outcome of a screening hybridization assay that used to be laborious and time-consuming, as individual molecules had to be cloned and analyzed. In this study, we combined another technique, differential display, as an additional screening step to identify upregulated genes of HCC. In this method, differential

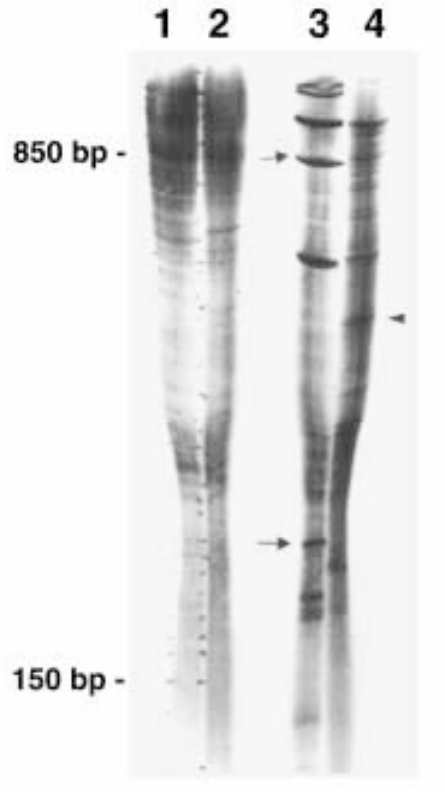

Fig. 2. Display of cDNA fragments on a 6% polyacrylamide sequencing gel after consecutive rounds of subtractive hybridization of experimental (left panel) and control (right panel). Lanes 1 and 3 represent cDNA fragments after complete rounds of subtractive hybridization, whereas lanes 2 and 4 show as initial unsubtracted cDNA fragments. To visualize the cDNA, it was amplified in the presence of [α-^{35}S] labeled dATP and exposed to an X-ray film. The arrows (lane 3) indicate examples of the enriched ϕX174/*Hae*III DNA bands, and the arrowhead (lane 4) shows an abundant cDNA fragment existing in the initial cDNA population which was depleted by subtraction.

gene expression is visualized by comparison of the different intensities of cDNA fragments after electrophoretic separation on a sequence gel. After conversion into cDNA using reverse transcriptase, all gene products can be amplified as distinct, primer-dependent groups of cDNAs. Although this approach has been shown to be feasible and reliable for identification of potential HCC markers, a number of concerns should be taken.

2. Poly(A^+) RNA should be extracted either from fresh tissue or from promptly frozen tissue, because intact mRNA is essential throughout the whole experiment.

Fig. 3. Northern blot analysis of 20 µg total RNA hybridized with a ferritin-H probe (Fe-H). Lane 0, 1, 2, and 3 represent RNA derived from rat liver induced with DENA for 0, 1, 2, and 3 mo respectively. The intensity of cDNA bands was quantitatively determined with the Phosphorimager and standardized by comparison to ^{28}S rRNA (28 S).

3. Effective subtractive hybridization needs a thorough cleavage by combined treatment with *Rsa*I and *Alu*I/*Rsa*I, thus causing the number of large cDNA fragments to be limited. This will ensure, on the one hand, that the majority of cDNA fragments with appropriate lengths is efficiently amplified by PCR. On the other hand, this will warrant more than one cDNA fragment from a given mRNA molecule to participate in a hybridization reaction, and therefore, increase the probability of being identified, especially for these low abundant genes.
4. Designing and testing the ligation of two specific adapter/primers to two cDNA populations should be carried out in a manner as to prevent cross-amplification that occurs among the experimental cDNA populations and thus avoid possible contamination.
5. Biotinylation of driver DNA and subsequent incubation with straptavidin-enhanced depletion of common cDNAs, therefore minimizing the number of cDNAs to be further analyzed.
6. Rounds of subtraction should be no more than three, to reduce the possibility that these low abundant cDNAs will be eliminated, whereas nonspecific ones, even artificial PCR products, could be introduced ***(17)***.
7. For display the subtraction-enriched cDNA bands, 0.5 µ*M* [α-^{35}S] dATP should be used, and concentration of nonradiolabeled dATP was lowed from 200 to 20 m*M* in order to obtain a high specificity.

Acknowledgments

We gratefully thank Dr. A. Bosma for histopathologic examination of HCC specimens, and Ms. J. Welsh, Dr. X. W. Wang, and X. L. Zhou for their assistance with the manuscript.

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11

Measurement of Protein Expression of p53, p21^{WAF1}, and Rb in Patients with Surgically Treated Hepatocellular Carcinoma by Using Catalyzed Signal-Amplification System

Takuji Naka, Tetsuya Kaneko, and Nobuaki Kaibara

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common human malignancies in the world and it is especially prevalent in Asia and Africa ***(1)***. Some tumors are suitable for resection, however, there is a very high rate of recurrence ***(2)***. Infection with hepatitis B or C virus, alcoholic cirrhosis, and ingestion of aflatoxin B1- contaminated food are important risk factors for HCC ***(3,4)***, but the exact molecular mechanism of tumorigenesis is still unclear.

Recent studies demonstrate that allelic imbalance or loss of heterozygosity occurs frequently on chromosomes 1p, 4q, 5q, 10q, 11p, 13q, 16q, and 17p in human HCC ***(5–9)***. Allelic loss on chromosome 17p, which harbors the p53 tumor-suppressor gene, is of particular importance and it has been observed to be lost in many human tumors, including HCC ***(8,10)***. Furthermore, allelic loss on chromosome 16 and changes in tumor-suppressor genes such as retinoblastoma (Rb) and p53 have been reported to be associated with tumor progression ***(10,11)***. Previous researchers characterized the function of p53 in the downregulation of cell growth by controlling cell entry into the S phase of the cell cycle.

Our initial immunohistochemical studies investigated the expression of important cell-regulatory gene protein products, such as p53, p21(WAF1), Rb, and cyclin D_1, in HCCs from a series of surgically resected patients. The standard streptavidin–biotin complex method was used and we found that the proportion of HCCs exhibiting positive immunostaining was much lower than

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

expected and the results were judged to be negative or unevaluable. Rather than accept that these initial findings were due solely to a lack of protein expression from these genes in the HCCs, we also speculated that there could be a technical problem with regard to the sensitivity of some immunohistochemical reactions in certain tissues when using the standard streptavidin–biotin complex method.

An amplification method in avidin–biotin systems for histochemical staining, a catalyzed signal–amplification system, has been recently reported ***(12,13)***. This new system has been reported to increase the sensitivity of a variety of histochemical reactions, including immunohistochemistry, enzyme immunoreactivity assays, and detection of *in situ* hybridization signals ***(14)***. The catalyzed signal-amplification system is achieved by the deposition of biotin-conjugated tyramine onto the specimen of biotinylated phenol, resulting in an amplification of the number of biotin molecules available for binding to the next reagent, streptavidin–peroxidase (*see* **Fig.1A–F** for a schematic summary of assay system reactions).

When we applied this catalyzed signal-amplification system to immunohistochemical detection of mutant p53, p21(WAF1), Rb, and cyclin D1 protein in our series of cases of surgically resected HCC, the immunostaining sensitivity was greatly increased, and our results showed that positive immunostaining for mutant p53 protein expression was a significant indicator of tumor progression and poor prognosis **(Fig. 2)** and p21 protein expression is induced in a p53-dependent manner, and they also suggested that Rb protein expression may be regulated to some extent by p21 in HCC.This suggests that gene therapy for HCC using these genes will be possible in the future. We introduce and describe our method of immunohistochemical staining using the catalyzed signal-amplificationsystem in this series of HCC cases.

2. Materials

1. All specimens are sections of surgically resected HCC. Following routine surgical histopathological methodology, all HCC specimens as soon as possible after surgical resection, should be fixed in 10% neutral-buffered formalin for several days, then embedded in paraffin. Using these blocks of paraffin-embedded tumor, cut 4-μm-thick sections of tissue on to poly-L-lysine-coated slides for immunohistochemistry.
2. Hydrogen peroxide: 3% and 8% hydrogen peroxide in water.
3. Protein block: serum-free protein in PBS.
4. 10 m*M* citrate buffer (pH 6.0) (stored at room temperature).
5. Phosphate-buffered saline (PBS): 0.05*M* phosphate buffer (pH 7.4) containing 0.145*M* sodium chloride (stored at room temperature).
6. Protein block: serum-free protein in PBS with 0.015*M* sodium azide (DAKO, Carpinteria, CA).
7. Primary antibody: anti-p53 monoclonal antibody (BP53-12; Novocastra Ltd.

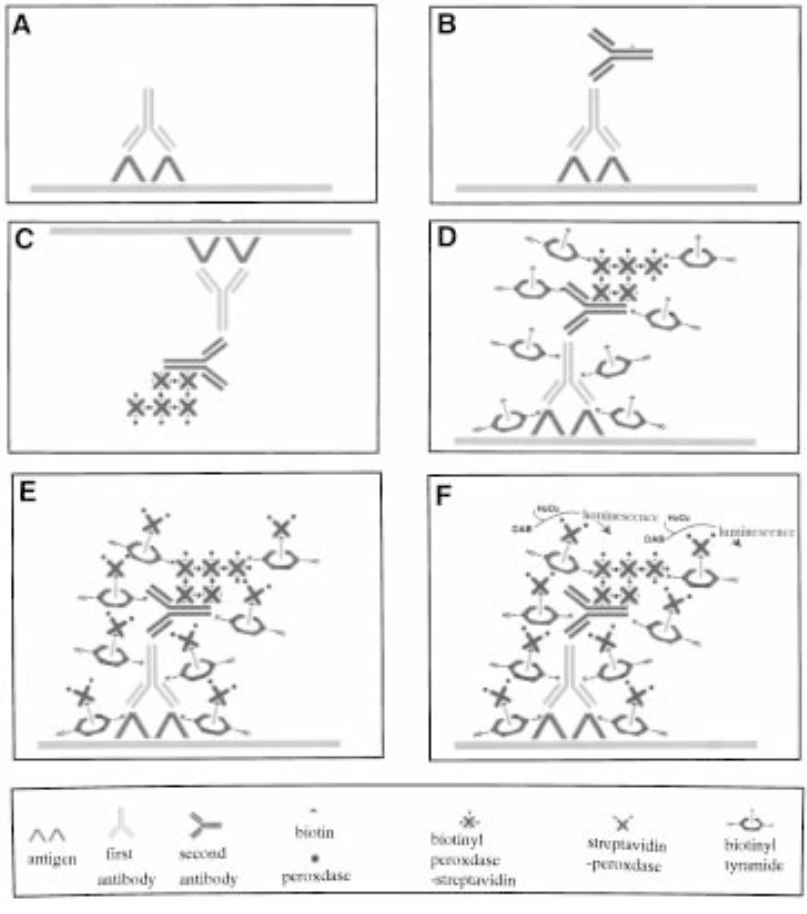

Fig.1. (A) Primary antibody–antigen complex. (B) Biotinylated secondary antibody combines with primary antibody. (C) Biotinylated peroxidase–streptavidin complex combines with the biotinylated secondary antibody. (D) In the presence of peroxidase, biotinylated tyramide will be changed to be radical products after reaction with peroxidase. The radical products are extremely unstable and they easily adhere to amino acids, which are located around antigenic sites. (E) A large amount of peroxidase–streptavidin combines with the biotin–tyramide complex. (F) Luminescence will be completed using the DAB substrate in the presence of large amounts of the peroxidase–streptavidin and biotin–tyramide complex.

Newcastle, UK) at a 1 : 50 dilution. Anti-p21 monoclonal antibody: PM2G12 (PharMingen. San Diego, CA) at a 1 : 100 dilution. anti-Rb monoclonal antibody: PMG3-245 (PharMingen. San Diego, CA) at a 1 : 50 dilution. Anti-cyclin-D1 monoclonal antibody 5D4 (MBL. Tokyo, Japan) at a 1 : 50 dilution (all stored at 4°C).

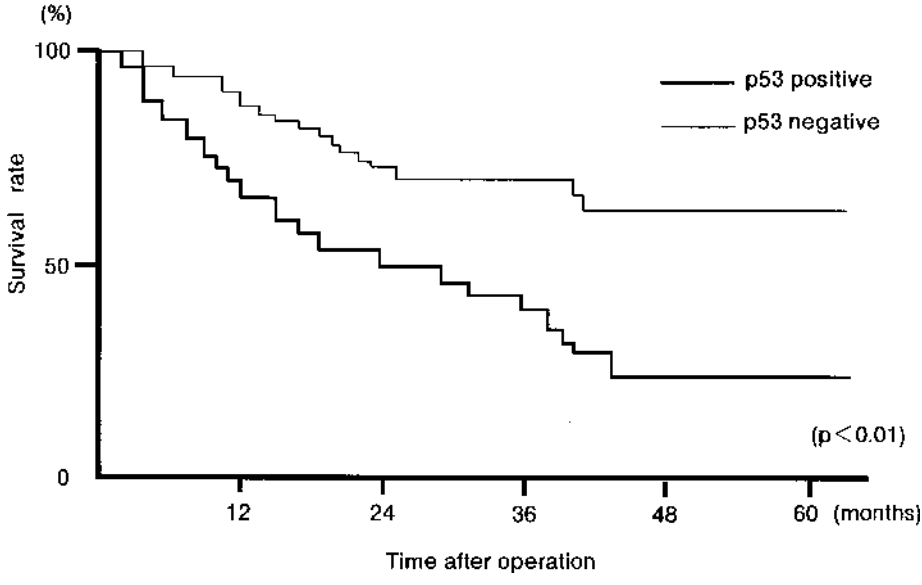

Fig. 2. Survival of patients in relation to mutant p53 protein status in HCC. Patients with tumors that exhibited positive mutant p53 protein immunostainning had a worse prognosis than those with negative p53 protein immunostainning ($p < 0.01$)

8. Link antibody: biotinylated rabbit anti-mouse immunoglobulins in Tris–HCl buffer containing carrier protein and 0.015 sodium azide (DAKO).
9. Streptavidin–biotin complex: streptavidin and biotin in PBS containing carrier protein and an antimicrobial agent (DAKO).
10. Amplification reagent: biotinyl tyramide and hydrogen peroxide in PBS containing carrier protein and antimicrobial agent (DAKO).
11. Streptavidin–peroxidase: streptavidin conjugated to horseradish peroxidase in PBS containing carrier protein and antimicrobial agent (DAKO).
12. DAB: 0.05*M* Tris–HCl buffer (pH 7.6) containing 10 mg of 3,3'-diaminobenzidine (DAB) and 100 μL of 5 % H_2O_2/100 mL.

3. Methods

1. Paraffin sections of the tumor are deparaffinized in xylene for 30 min and rehydrated in decreasing concentrations of ethanol (100%, 90%, 70%, 50%, distilled water, respectively).
2. Tissue sections are then treated three times with boiling for 5 min in 10 m*M* citrate buffer in a microwave oven (500 W) for antigen retrieval.
3. Tissue sections are left at room temperature in the buffer solution for 30 min and then rinsed with distilled water.
4. Quenching the endogenous peroxdase activity: The tissue slides are then immersed

in a solution of 3% hydrogen peroxide in methanol for 20 min, followed by gentle rinsing with distilled water and PBS.

5. Protein block: Apply enough drops of protein block to the specimen slides and incubate for 5 min at room temperature. Tap off excess protein block and wipe around the specimen.
6. Primary antibody: Apply enough drops of primary antibody (anti-p53, p21, Rb, and cyclin D1 monoclonal antibody) to cover each specimen. Incubate overnight at 4°C in a moist chamber. Rinse gently with PBS.
7. Link antibody: Apply enough drops of link antibody to cover specimen. Incubate 15 min at room temperature. Rinse gently with PBS.
8. Streptavidin–biotin complex: Apply enough drops of streptavidin–biotin complex to cover specimen and incubate for 15 min at room temperature. Rinse gently with PBS.
9. Amplification reagent: Apply enough drops of amplification reagent to cover specimen. Incubate 15 min at room temperature. Rinse gently with PBS.
10. Streptavidin–peroxidase: Apply enough drops of streptavidin–peroxidase to cover each specimen and incubate 15 min at room temperature. Rinse gently with PBS.
11. Peroxidase activity: Immerse specimen slides in a bath of DAB for about 5 min and then rinse the slides gently with distilled water.
12. Methyl Green counterstain (or Hematoxylin counterstain): Immerse the specimen slides in a bath of methyl green (or hematoxylin).
13. Mounting**:** Immerse the specimen slides in increasing concentrations of ethanol (50%, 70%, 90%, 100%, respectively) and then xylene for 20 min. Mount the specimens with a nonaquous mounting medium and coverslip.

4. Notes

By using the catalyzed signal-amplification system, positive immunostaining for Rb, p21, and mutant p53 protein was detected in 58%, 33%, and 37% of the HCCs, respectively **(Fig.3)**. These results showed that this amplification system has significantly increased the sensitivity for demonstration of these protein products in HCC tissue.

Several important points concerning general application of this method are as follows:

1. Survival of the tissue antigen for immunological staining may depend on the type and concentration of fixative, on the fixation time, and on the size of the tissue specimen to be fixed. It has been hypothesized that formalin and other aldehydes crosslink proteins around antigenic sites, resulting in a masking effect that interferes with binding of the antibody ***(15)***. Tissues fixed for brief periods of time may show relatively well-preserved antigenicity. It is important to maintain optimal, standard fixation conditions whenever possible in order to obtain reproducible staining.
2. A potential problem of the increased sensitivity afforded by this catalized signal-amplification system is the possibility of increased nonspecific background staining due to endogenous avidin-binding activity or endogenous peroxidase activity; it is therefore necessary to carefully control the dilution of primary antibody and DAB. It

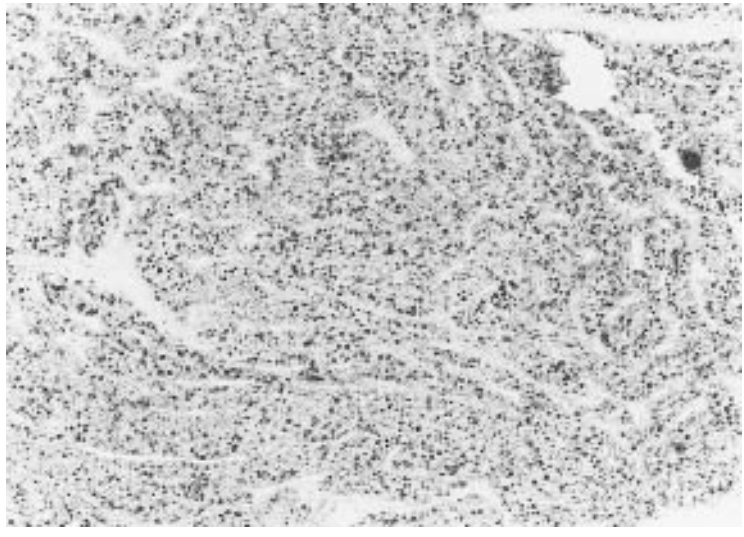

Fig. 3. Black-and-white photograph of positive immunostaining for mutant p53 protein in HCC. Positive staining for protein expression was seen in the nucleus of the tumor cells (magnification: X40).

is also important to use both positive and negative control reagents and specimens.

3. It is necessary for the tissue specimen sections to be boiled for three separate 5-min intervals in 10 m*M* citrate buffer in a microwave oven (500 W) for antigen retrieval after deparaffinization in xylene and rehydration in ethanol ***(16)***.
4. It is important not to allow the tissue specimen sections to dry out during the staining procedure, as this may cause increased nonspecific staining. Cover any tissue specimen slides exposed to drafts and incubate them in a moist chamber. If prolonged incubations are used, place the tissue specimen slides in PBS or distilled water.

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IV

HCC Gene Therapy

12

Gene Therapy Vectors Harboring AFP Regulatory Sequences

Preparation of an Adenoviral Vector

Shuichi Kaneko and Taiki Tamaoki

1. Introduction

A goal of cancer research is to develop therapies that can selectively kill tumor cells without adversely affecting normal cells. In the case of hepatocellular carcinoma (HCC), there is a possibility that this goal may be achieved by introducing a cytotoxic gene under the control of transcriptional regulatory sequences of the α-fetoprotein (AFP) gene. The expression of the therapeutic gene should be limited to AFP-positive cells (i.e., HCC), so that only tumor cells will be eliminated without harming normal cells. In this article, we will first review the main features of human AFP regulatory sequences with respect to tumor-specific transcriptional activity. We then describe details of an adenoviral vector carrying the human AFP regulatory sequences used for HCC-directed gene therapy.

1.1. Regulatory Sequences of AFP Gene

Transcription of the human AFP gene is controlled positively by the promoter and the enhancer ***(1–3)*** and negatively by an element called silencer ***(4)*** **(Fig. 1)**. The AFP promoter is a 200-bp region immediately upstream of the AFP gene ***(5–8)*** that is regulated by hepatocyte nuclear factor 1 (HNF1), nuclear factor 1 (NF1) and CCAAT/enhancer binding protein (C/EBP) **(Fig. 1)**. The human AFP promoter is upregulated by dexamethasone through a

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

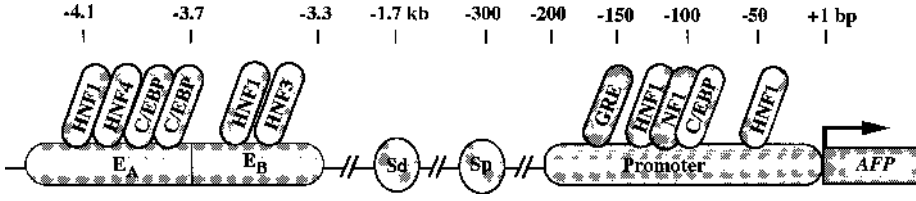

Fig. 1. Transcriptional regulatory regions of the human α-fetoprotein gene. E_A, enhancer domain A; E_B, enhancer domain B; Sd, distal silencer; Sp, proximal silencer.

glucocorticoid responsive element (GRE) ***(5)***. The AFP promoter is highly specific to HCC, but its transcriptional activity is relatively weak. The human AFP enhancer is present between 3 and 4.9 kb upstream of the transcription initiation site of the AFP gene ***(2)***. At least four transcription factors, including HNF1, HNF3, HNF4, and C/EBP, are known to bind to the human AFP enhancer **(Fig. 1)**. The human AFP enhancer, like the promoter, shows HCC specificity, which may result largely to the action of HNF1 ***(2)***. ATBF1 also binds to the HNF1 site in competition with HNF1 to downregulate AFP enhancer activity ***(9,10)***. No HNF1 site has been found in mouse, and rat AFP enhancers, although all other transcription factor binding sites are shared by the human, mouse and rat AFP enhancers. The rodent AFP enhancers also differ from the human AFP enhancer in that they are located in three widely separated regions that exhibit different tissue specificities ***(11,12)***. In HCC and fetal liver, the enhancer is believed to be crucial for high levels of AFP expression. Most past studies of gene therapy of HCC have used the human AFP enhancer to stimulate the AFP promoter, although one of the three mouse AFP enhancer regions having liver specificity has also been used successfully ***(13)***. Two silencer regions have been identified upstream of the human AFP gene, one at –0.31 kb and the other at –1.75 kb, with the latter exhibiting much stronger suppressive activity than the former ***(5)*** **(Fig. 1)**. The function of the AFP silencer is to prevent the AFP enhancer from stimulating the AFP promoter. This inhibitory effect is manifested only when the silencer is situated downstream of the enhancer and upstream of the promoter. In human HCC cell lines, the silencer activity is inversely correlated with the level of AFP expression, suggesting that the silencer is involved in determining the level of AFP expression in HCC. The silencer also plays a major role in suppressing AFP expression during liver ontogeny ***(14,15)***, although this is not the only mechanism involved in developmental regulation of the AFP gene ***(16)***. The possibility exists that the silencer may serve as a molecular switch to redirect the action of the AFP enhancer from the AFP promoter to the albumin promoter as the liver matures ***(17)***. In gene therapy of

HCC, the use of the silencer is desirable, as it will ensure that the therapeutic gene expression is restricted to AFP-positive HCC cells.

1.2. Adenoviral Vectors Harboring AFP Regulatory Sequences

Adenoviral vectors gained considerable interest in the early 1990s owing to their capacity to infect quiescent cells with high efficiency. However, it soon became apparent that transgene expression is transient and most of the transduced cells rapidly disappear. These results were disappointing for the purpose of treating hereditary diseases. For the treatment of cancer, however, transient expression may be acceptable if it occurs at a high level and in a significant proportion of cancer cells. Adenoviral vectors can infect nontumorous cells as well as tumor cells. This would potentially cause toxicity in nontumorous tissues, especially damaged ones, such as cirrhotic liver. To achieve specific killing of HCC, adenoviral vectors carrying AFP regulatory sequences have been constructed ***(18–21)***. We describe the details of the preparation of an adenoviral vector designed to express the herpes simplex virus thymidine kinase (HSV-TK) gene (22) under the control of the 4.9-kb AFP 5'-regulatory sequence (*see* **Note 1**). Other viral vectors containing AFP-regulatory sequences are also discussed (*see* **Notes 2–4**).

2. Materials

2.1. Plasmids

1. pAF4.9-CAT ***(2)***.
2. Cla I fragment derived from Ad-dl327 ***(23,24)***.
3. pAvCMVTK1 ***(23,24)***.

2.2. Enzymes and Kits

1. Asc I (10 U/µL, New England Biolabs, Beverly, MA).
2. Nhe I (5 U/µL, New England Biolabs)
3. Bst BI (20 U/µL, New England Biolabs).
4. Spe I (3 U/µL, New England Biolabs).
5. Xmn I (20 U/µL, New England Biolabs).
6. T4 DNA ligase (2000 U/µL, New England Biolabs).
7. Pfu DNA polymerase (Stratagene, La Jolla, CA).
8. GENECLEAN II kit (BIO 101, La Jolla, CA).
9. Calcium phosphate mammalian cell transfection kit (5 Prime 3 Prime Inc., Boulder, CO).
10. TE buffer: 10 m*M* Tris–HCl, pH 7.5, 1 m*M* EDTA.

2.3. Cells and Reagents

1. DH5α *E. coli* (Gibco-BRL, Rockville, MD).
2. 293 cells (CRL 1573, ATCC, Rockville, MD).
3. Complete medium (IMEM (BioWhittaker, Walkersville, MD), 10% fetal bo-

vine serum, 2 m*M* glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, 1% [w/v] Fungizone).
4. Low-melting-point agarose (SeaPlaque) (FMC, Rockland, ME).
5. Minimal Essential Medium (MEM) (BioWhittaker).
6. 1.25 g/mL CsCl solution.
7. 1.33 g/mL CsCl solution.
8. 1.40 g/mL CsCl solution.
9. 1X dialysis buffer: 10 m*M* Tris–HCl, pH 7.4, 1 m*M* $MgCl_2$, and 10% (v/v) glycerol.

3. Methods

To generate recombinant adenovirus, 293 cells are transfected with the *Cla*I fragment of Ad-dl327 (adenovirus fragment) **(Subheading 3.1.2.)** together with an adenoviral vector DNA **(Subheading 3.1.)**. The recombinant virus produced is selected by plaque formation **(Subheading 3.2.2.)** and, subsequently, purified **(Subheading 3.2.3.)**.

3.1. Preparation of Adenoviral Vector DNA (pAvAFPTK1) (Fig. 2)

3.1.1. Preparation of a pAvCMVTK1 Fragment

1. Digest pAvCMVTK1 DNA with Asc I and Nhe I.
2. Electrophorese the reaction mixture on a 1% agarose gel and cut out the portion containing the larger fragment of the two DNA products.
3. Extract the DNA from the gel using GENECLEAN II (according to the manufacturer's protocol), and elute DNA with TE buffer.

*3.1.2. Preparation of a Small AFP-Promoter Fragment by PCR (see **Note 5**)*

1. Amplify a small AFP promoter of pAF4.9-CAT using polymerase chain reaction (PCR) with two primers having restriction enzyme sites. A sense primer was designed to locate upstream of spe I site (–65 bp), as indicated by the dashed line in **Fig. 2**, and to have *Asc*I and *Bst*BI sites at the 5' terminal. An antisense primer was designed to start from *Hind*III site (+29 bp) and to have *Nhe*I site at the 5' terminal.
2. Electrophorese a portion of the reaction mixture on a 1% agarose gel to verify the size of amplified DNA.
3. Purify the PCR-amplified DNA from the remaining reaction mixture by shaking with an equal volume of phenol–chloroform and precipitating the DNA with EtOH. Dissolve the DNA in TE buffer.
4. Digest the DNA with Asc I and Nhe I. Electrophorese the product on a 1% agasose gel, purify with GENECLEAN II, and elute DNA with TE buffer.

3.1.3. Preparation of a BstBI (–4.9 kb) and SpeI (–65 bp) AFP Fragment from pAF4.9-CAT

1. Digest pAF4.9-CAT with *Bst*BI and *Spe*I.

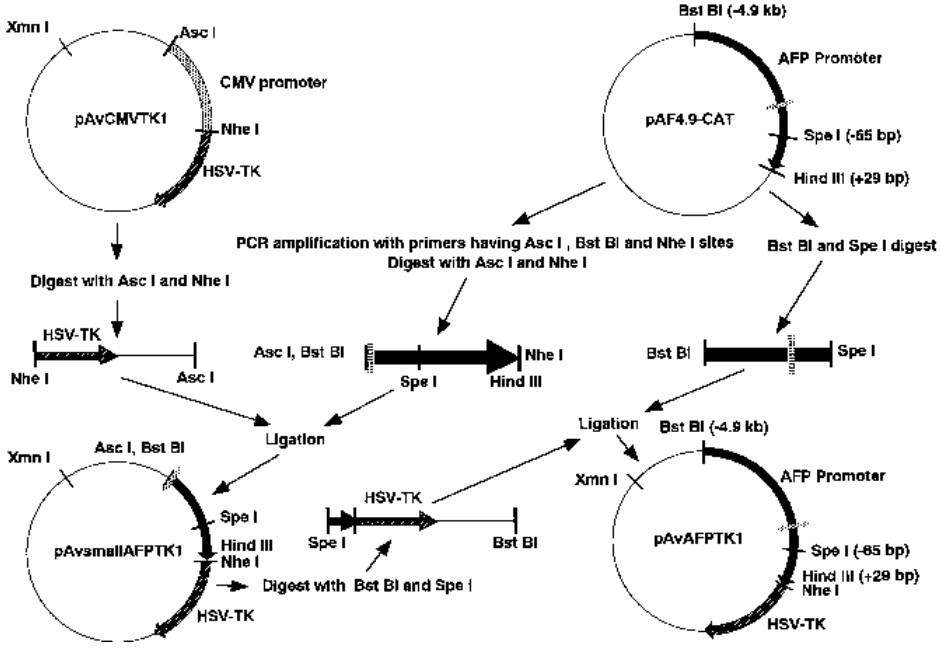

Fig. 2. Construction of adenoviral vector DNA harboring a 4.9-kb AFP promoter.

2. Electrophorese the sample on a 1% agarose gel and cut out the larger of the two DNA fragments.
3. Extract DNA from the gel using GENECLEAN II and elute DNA with TE buffer.

3.1.4. Construction of pAvAFPTK1 (Adenoviral Vector DNA)

1. Ligate the Asc I/Nhe I fragment of pAvCMVTK1 **(Subheading 3.1.1.)** and the *Spe*I/*Hind*III AFP fragment from pAF4.9-CAT **(Subheading 3.1.2.)** using T4 DNA ligase to form pAvsmallAFPTK1.
2. Transform DH5α *E. coli* with the above reaction mixture. Pick and grow the transformant and prepare the plasmid DNA, pAvsmallAFPTK1.
3. Digest pAvsmallAFPTK1 with *Bst*BI and *Spe*I and electrophorese on a 1% agarose gel to separate the larger of the two DNA fragments. Purify it with GENECLEAN II and elute DNA with TE buffer.
4. Ligate the DNA and the *Bst*BI/*Spe*I AFP fragment **(Subheading 3.1.3.)** using T4DNA ligase to form pAvAFPTK1.
5. Transform DH5α *E. coli* with the above reaction mixture. Pick and grow the transformant and prepare the plasmid DNA, pAvAFPTK1.
6. Perform sequence analysis of the AFP DNA in pAvAFPTK1.

3.2. Generation of Recombinant Adenovirus

3.2.1. Cotransfection of the Adenoviral Vector DNA and an Adenovirus Fragment

1. Grow 293 cells in the complete medium to 80% confluency (*see* **Note 6**).
2. Transfect the cells with pAvAFPTK1 linearized by *Xmn*I digestion and the large *Cla*I Ad-dl327 fragment using the calcium phospate mammalian transfection kit.
3. Overlay the cells with 1% SeaPlaque prepared by mixing 2% SeaPlaque and 2X MEM at a ratio of 1 : 1.
4. Incubate the cells at 37°C in humidified 5 % CO_2 atmosphere.
5. Repeat the overlay procedure on d 5 and 10.
6. Plaques are formed in 2–3 wk.

3.2.2. Amplification of Recombinant Adenovirus from Plaques on 293 Cells

1. Select and harvest plaques from the transformation plate.
2. Freeze and thaw five times to lyse the cells and release the virus. Remove cellular debris and agarose by centrifugation.
3. Infect 293 cells with the crude lysate.
4. Harvest cells when the cells show the cytopathic effect with a few cells floating in the medium.
5. Freeze and thaw cells five times.
6. Remove cell debris by centrifugation.

3.2.3. Purification of Recombinant Adenovirus

1. Place 11 mL of 1.25 g/mL CsCl in a 39-mL Beckman ultraclear sealable centrifuge tube (#344326).
2. Underlay 11 mL of 1.40 g/mL CsCl.
3. Overlay 17 mL of the lysate.
4. Centrifuge at 167,000*g* for 60 min in a Beckman VAC-50 rotor.
5. Collect the lower of the two opalescent bands that contains the intact recombinant virus by side puncture. The virus is then purified by second centrifugation as follows.
6. Place 24 mL of 1.33 g/mL CsCl in a 39-mL Beckman ultraclear sealable centrifuge tube (#344326).
7. Overlay 6 ml of the above virus (**Subheading 3.1.4., step 5**).
8. Centrifuge at 167,000*g* for 18 h in a Beckman VAC-50 rotor.
9. Collect the opalescent band.
10. Dialyze against 1X dialysis buffer.
11. Recover the virus (*see* **Note 7**).

3.2.4. Titration of Recombinant Adenovirus (*Notes 8* and *9)*

1. Grow 293 cells in six-well plates to 80% confluency.
2. Prepare 1 : 10 serial dilutions of the virus solution.
3. Inoculate the cells with the above.
4. Overlay the cells with 1% SeaPlaque prepared by mixing 2% SeaPlaque and 2X

MEM at a ratio of 1 : 1.
5. Repeat the overlay procedure on d 5 and 10.
6. Read plaques in wells under microscope.

4. Notes

1. Anti-tumor effect of the recombinant adenoviral vector. AFP-producing HuH7 cells and non-AFP-producing SK-Hep-1 cells were infected with Av1AFPTK1 and Av1TK1 which contain the HSV-TK gene under the control of the 4.9–kb AFP regulatory sequence and the Rous sarcoma virus promoter, respectively, at multiplicity of infarction (MOIs) 10 and 100, and HSV-TK expression was determined by analyzing phosphorylated ganciclovir (GCV) **(Table 1)**. In HuH7 cells infected with either recombinant virus, the TK activity was expressed in a dose-dependent manner. In SK-Hep-1 cells infected with Av1AFPTK1, no HSV-TK activity was detected at both MOIs, but infection with Av1TK1 resulted in expression of HSV-TK activity in a dose-dependent manner. These results show tumor cell-specific expression of the HSV-TK gene from Av1AFPTK1 in vitro. In an animal model, HuH7 and SK-Hep-1 cells were injected into athymic mice, tumors developed from these cells received Av1AFPTK1 or Av1TK1 by direct injection, and the mice were injected with GCV intraperitoneally. All 10 HuH7 tumors that received either Av1AFPTK1 or Av1TK1 completely regressed **(Fig. 3A,B)**. In contrast, all five tumors without adenoviral injection continued to grow. SK-Hep-1 tumors that received Av1AFPTK1 did not regress following GCV treatments, although those infected with Av1TK1 regressed completely. These results show that the recombinant adenovirus which expresses the HSV-TK gene under the control of the 4.9-kb AFP regulatory sequence was effective in eliminating the AFP-producing tumors in vivo. Two issues may be raised in relation to the results described. First, these experiments were performed in human HCC-derived cells established in nude mice. Although the efficacy of the tumor-specific expression system was clearly demonstrated, the antitumor effect may not be strong enough by the lack of antitumor immunity in nude mice. The usefulness of the strategy should be tested in animals with normal immunological competency. Second, the level of expression of the HSV-TK gene achieved by AFP regulatory sequences was low compared with that driven by the RSV promoter. Further work is necessary to evaluate whether the level of gene expression attained by Av1AFPTK1 is high enough to be useful in clinical settings.
2. Enhanced gene expression the using Cre–loxP system. Sato et al. ***(25)*** used the Cre–loxP system ***(26–28)*** to enhance the level of gene expression under the control of the AFP-regulatory sequences. In this work, two recombinant adenoviral vectors were constructed, one containing the Cre recombinase gene linked to AFP regulatory sequences to express Cre in HCC cells producing AFP and the other carrying the *lacZ* gene linked to a powerful hybrid promoter termed CAG ***(29)***. A stuffer sequence with the loxP element at each end was inserted between the CAG promoter and the *lacZ* gene to prevent the CAG promoter from initiating transcription of the *lacZ* gene. When HuH-7 cells were infected with theses two

Table1
HSV-TK Gene Expression by Recombinant Adenoviral Vectors

Adenovirus	MOI	HuH7	SK-Hep-1
Av1AFPTK1	10	24cpm	0cpm
Av1AFPTK1	100	128cpm	0cpm
Av1TK1	10	849cpm	110cpm
Av1TK1	100	17,472cpm	5,270cpm

Note: AFP-producing (HuH7) and AFP-non-producing (SK-Hep-1) cells were infected with either Av1AFPTK1 or Av1TK1. HSV-TK activity in the infected cells was determined by TK enzyme assay using ^{3}H-labeled GCV ***(18)***.The amount of phosphorylated GCV was measured by scintillation counting.

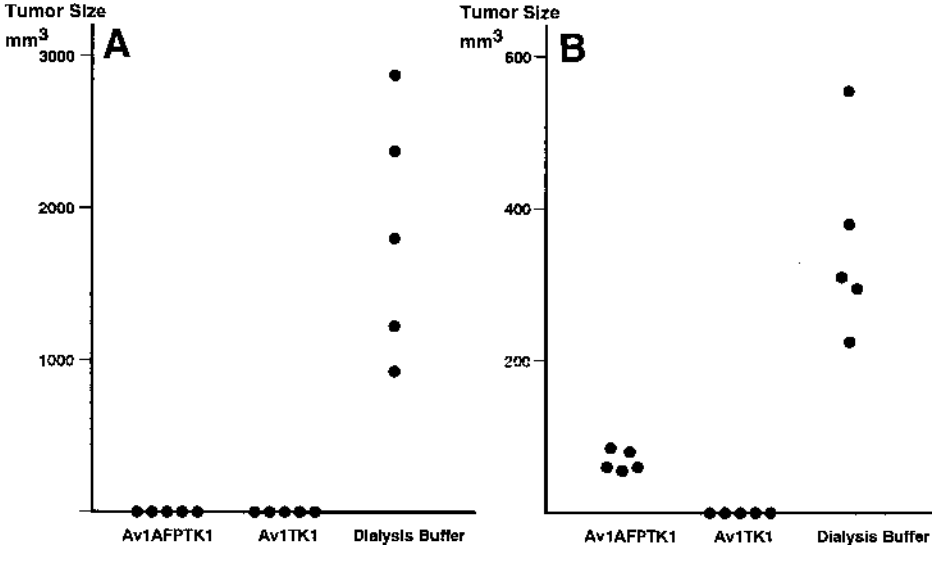

Fig. 3. Effect of injection of recombinant adenoviruses into tumors, followed by GCV administration in nude mice. Av1AFPTK1, Av1TK1, or dialysis buffer was injected into tumors derived from AFP-producing HuH7 cells (A) and AFP-nonproducing SK-Hep-1 cells (B), and GCV was injected into mice intraperitoneally. Tumor sizes were measured 10 d after cessation of GCV treatment ***(18)***.

types of recombinant virus, Cre recombinase was expressed under the control of the AFP regulator, which, in turn, recognized the loxP sequence to delete the stuffer sequence, allowing the CAG promoter to initiate transcription of the *lacZ* gene. The level of *lacZ* activity thus expressed in HuH7 cells was 50-fold higher than that expressed by recombinant viruses carrying the *lacZ* gene under the direct control of the AFP-regulatory sequences. In non-AFP-producing cells, Cre was not expressed and, consequently, no appreciable *lacZ* expression was observed.

Thus, this system enables enhanced gene expression without losing specificity to HCC. Further studies using a therapeutic gene such as the HSV-TK gene in animal models are desired.

3. Retrovirus. Recombinant retroviruses carrying the varicella–zoster virus thymidine kinase gene under the control of the 5.1-kb AFP 5'-flanking sequence has been shown to cause cytotoxicity mediated by 6 methoxypurine arabinonucleoside in AFP-positive HCC cells in vitro ***(30)***. Similarly, recombinant retroviruses having the HSV-TK gene under the control of the 0.3-kb AFP promoter caused GCV-mediated killing of AFP-positive HCC cells ***(31)***. In an animal model, subcutaneous tumors generated in nude mice by implanting AFP-positive PLC/PRF/5 cells previously transduced with retroviruses carrying the HSV-TK gene under the control of the AFP promoter linked to the AFP enhancer domain B were highly sensitive to GCV injected into the mice intraperitoneally ***(32)***.
4. Adeno-associated virus (AAV). AFP-positive HCC cells have been shown to be killed by infection of recombinant AAV carrying the HSV-TK gene under the control of the human albumin promoter and the human AFP enhancer domain B followed by GCV treatment in vitro ***(33)***. In nude mice, subcutaneous tumors arising from recombinant AAV-transduced PLC/PRF/5 cells showed marked regression after intraperitoneal injection of GCV ***(34)***. Tumors derived from untransduced cells also regressed by direct injection of the recombinant AAV into tumors followed by intraperitoneal injection of GCV ***(34)***.
5. Preparation of AFP fragments by PCR. By the use of PCR with primers having restriction enzyme sites, molecular cloning of a small AFP fragment can be done relatively easily. Sequence analysis of the PCR product should be performed to ensure that no nucleotide is altered during PCR.
6. 293 Cells are sensitive to drying, low temperatures, pH changes, and excessive mechanical manipulation.
7. Contamination of wild-type adenovirus. The viral stock should be examined for the presense of E1a to ensure that it is not contaminated with the wild-type adenovirus.
8. The kinetics of plaque formation depends on factors which are not well understood. Plaques generated by transfection of DNA take longer to form (2–3 wk) than those by infection of intact virions (1–2 wk). Therefore, reading of the titer should be standardized with regard to the time. Generally, mutant viruses deficient of the E1 region form well-developed plaques in 2 ws to allow determination of titers.
9. In reading titers, examine plaque morphology to avoid counting artifacts.

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13

Advantages and Disadvantages of Multiple Different Methods of Adenoviral Vector Construction

Prem Seth and James Higginbotham

1. Introduction

Adenoviruses are medium-sized nonenveloped DNA tumor viruses whose genome consists of a linear double-stranded DNA molecule of about 36 *K*b (reviewed in **refs. *1–3***). Adenoviruses have icosahedral geometry with a diameter of approx 70–100 nm. Currently, over 100 different mammalian and foul adenovirus (Ad) serotypes have been characterized, with 49 of them being human serotypes. The most extensively characterized serotypes are 2, 5, and 12, with type-5-based vectors being the most popular. Ad virus transcription, replication, and packaging have been extensively studied and take place in the nucleus of infected cells, with transcription and replication requiring both cellular and viral proteins. Expression of the Ad genome is divided into two phases: early, which occurs before DNA replication, and late, which occurs after the initiation of DNA replication. Transcription during the early phase of adenoviral infection involves the highly orchestrated expression of four pol II-dependent regions, E1, E2, E3, and E4, which each code for multiple mRNAs. E1 is further divided into two transcriptional units E1A and E1B. E1A coordinates transcriptional control of the virus, whereas E1B gene products influence the early to late transition by coordinating viral to cellular mRNA metabolism and host protein shutoff. Additionally, the E1B region obstructs the apoptotic signals induced by E1A. The E2 region codes for three proteins essential for viral DNA replication; terminal protein precursor, DNA polymerase, and DNA binding protein. E3 is not required for Ad replication but does play the important role of suppressing the immune response to virally infected cells in vivo.

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

E4 enhances late viral gene expression and facilitates the shutoff of host protein synthesis. The late phase of viral gene expression is directed by the regulatory proteins of E4. Most of the late transcripts (L1, L2, L3, L4, and L5) are initiated by the major late promotor and are differentially spliced to yield almost all of the viral structural proteins. All five late mRNA families have the same 5' tripartite leader sequence and each family shares a common cleavage and poly-A site but with different intron splicing. The major Ad capsid components consist of hexon (L-3), penton base (L-2), and fiber (L-5) (reviewed in **refs. *1–3***).

Adenovirus vectors are currently the most efficient gene-transfer vehicles today because of their ability to transduce many cells types with high efficiency both in vitro and in vivo. A number of features unique to adenoviruses contribute to their utility as gene-transfer vectors, including the following: highly stable viral particles that can be concentrated to high titers [moderate scale production typically yields several milliliters of (2–5)X 10^{11} plaque-forming units/mL -{PFU/mL}]; the ability to easily manipulate the Ad genome using standard molecular biology techniques; Ad vectors are reasonably stable after cloning; Ad vectors exhibit extremely wide tropism; and Ad vectors are able to transduce dividing and nondividing cells without requiring pretreatment of the target cells with exotic growth factors or toxic agents for efficient transduction ***(3)***. Furthermore, Ad vectors can be engineered to be replication-competent or replication-defective, depending on the desired use of the vector. Ad can correctly package up to 105% of the wild-type genome, thus accommodating 1.8 kb of insert without concomitant deletion of viral sequences ***(4)***. In order to accommodate larger transgene inserts, Ad vectors have been constructed that delete much of the nonessential E3 region to construct a replication-competent vector with an maximum insert size of about 5 kb ***(5)***. The most commonly used Ad vectors are the first-generation $E1^{-}/E3^{-}$ replication-defective adenoviruses that are propagated on cell lines supplying E1 functions in *trans.* It has been demonstrated that the left inverted terminal repeat (nucleotides 1–103 of Ad5) and the viral packaging signal (nt 194–358) are required in *Cis* for viral replication ***(6)***. Additionally, the pIX (nt 3525–4088) is required for efficient packaging of full-length viral DNA and brings the maximum E1 deletion of about 3.2 kb. $E1^{-}/E3^{-}$- deleted vectors thus have an insert capacity of 8.1 kb. First-generation $E1^{-}/E3^{-}$ Ad vectors are normally cloned and propagated on 293 cells, a human embryonic kidney cell line transformed with about 4 kb of the left end of the Ad5 genome. The 293 cell line expresses both E1A and E1B but not the pIX, even though pIX sequences are present in E1B ***(7)***. A new transcomplementing cell line dubbed 911 has been generated from human embryonic retinoblasts transfected with a plasmid containing 5.7 kb from the left end of the Ad5 genome (nt 79–5789). The 911 cell-line-like 293 cells

exhibit enough homology with most current first-generation vectors that the formation of replication-competent Ad (RCA) is not excluded; however, this process usually requires many sequential passages of the vector or a negative selective pressure of a toxic transgene. There have been no reports of RCA generation in 911 cells ***(8)***.

Construction of first-generation Ad vectors is relatively labor intensive because of the scarcity of unique or useful restriction sites within the large Ad genome. Two methods of Ad vector construction have dominated for the last 10–15 yr: an in vitro ligation method ***(9–11)*** and a homologous recombination method in 293 cells ***(3,5,12,13)***. The first method of in vitro ligation has fallen out of favor in recent years. This method uses a cloning plasmid containing the left end of the Ad genome with the left ITR, the packaging signal, and E1A enhancer sequence (map units 0–1.3) and whole viral genomes. The transgene of interest is cloned into the cloning plasmid behind the viral sequences, heterologous promotor, intron, and polyadenylation site for maximum expression. The fragment of the cloning plasmid containing the viral sequences and transgene transcriptional unit are excised and ligated into the unique *Cla*I site of the viral genome (map unit 2.6), thus replacing part of the E1 region. The ligated DNA is then transfected into 293 cells to make recombinant virus. This method of construction is impeded because of the necessity of multiple rounds of plaque purification to remove contaminating wild-type and null viruses that arise as a result of incomplete restriction and religation. Recent improvements of the in vitro ligation have been reported that may bring this method of vector construction back in vogue ***(14,15)***. The improved in vitro ligation method of Okada uses Ad DNA–protein complexes that allow directional cloning of the transgene. The construction of Ad vectors using DNA–protein complexes is faster than in vivo homologous recombination method by about 5–7 d. Much of the improvement in the speed of DNA–protein complexes can be attributed to a 2 log increase in infectivity of the Ad DNA. An advantage of DNA–protein complexes is the lack of necessity of screening for the orientation of the transgene is obviated by directional cloning. Additionally, the DNA–protein method does not generate high backgrounds of wild–type or null vectors that have to be removed by multiple rounds of plaque purification. The DNA–protein complex method of Ad vector construction improves speed and efficiency of recombinant vector generation, but it is a more complex system. The DNA–protein complex method requires multiple rounds of CsCl gradients, desalting, digestion of the multiple cloning site (MCS), and removal of the small intermediate DNA fragment of the MCS. Additionally, the DNA–protein complex ''arms'' require dephosphorylation to reduce the ligation efficiency of contaminating viral sequences. The more recent in vitro ligation method of Mizuguchi and Kay capitalizes on the insertion of three unique restriction sites I-*Ceu*I, *Swa*I, and PI-*Sce*I into the E1 dele-

tion site of the vector plasmid containing a first-generation $E1^{-}/E3^{-}$ Ad genome. After cloning the transgene into a shuttle plasmid containing the *Ceu*I and PI-*Sce*I sites, the insert containing the transgene can then be easily ligated into the *Ceu*I and PI-*Sce*I sites of the Ad genome vector and digested with *Swa*I to reduce the production of the parental plasmid (null vector). Mizuguchi and Kay report that more than 90% of the subsequent transformants had the correct insert. The Ad vector was then generated by transfection of *Pac*I-digested linearized plasmid onto 293 cells. These improvements to the in vitro ligation method greatly reduce the background production of wild-type and null vector and, thus, reduce time-consuming screening of plaques. The more popular in vivo homologous recombination method of construction was initially developed as a two-plasmid system, with the first plasmid (pJM17) containing the Ad5 genome and additional bacterial sequences that exceeded the packaging capacity of the viral capsid ***(12)***. The second plasmid contains the left ITR, packaging signal, transgene transcriptional unit, and a region of overlapping sequence. This system was improved by building another plasmid containing the Ad genome without the packaging sequence that increases efficiency of the system by reducing background null-virus recombinants ***(5)***. Both plasmids are cotransfected into 293 cells, where homologous recombination yields recombinant virus. This system of construction is limited primarily by the low frequency of homologous recombination and the necessity of multiple rounds of plaque purification to remove contaminating null viruses. Recent improvements have been made in the homologous recombination method by performing the recombination event in yeast or bacteria, where the events occur with greater frequently ***(16–19)***. The use of yeast cells to perform the homologous recombination event is more efficient but requires specialized skills to isolate and manipulate yeast artificial chromosomes. The other system uses a nonconventional *Escherichia coli* strain and a multistep transformation for the first step. The *E. coli* strain (BJ5183recBCsbcBC) used in the homologous recombination step does not yield enough of the recombinant viral DNA for standard transfection on 293 cells and, thus, must be amplified in another *E. coli* strain to have enough DNA ***(17–19)***. Significant resources have been devoted to develop second-generation Ad vectors by making additional deletions in the E2 or E4 regions to reduce the low level viral replication due to leaky late-region gene expression in the first generation Ad vectors ***(20–22)***. Although the primary goal of reduced late-region gene expression was achieved, this was not sufficient to prevent the cellular and humoral responses toward the vector. More recently, Ad vectors have been developed that have most of the genome deleted with the exception of the *Cis*-acting elements required for packaging. These ''gutless'' vectors require a helper virus for propagation and have the advantages of extremely large cloning capacity (up to 35 kb) and removal of addi-

tional viral genes associated with initiation of the cellular and humoral immune responses toward the vector ***(23–26)***. Limitation of the ''gutless'' Ad vectors are the reduction in viral titer and the inability to remove all of the contaminating helper-virus. A recent study in which the helper-virus packaging sequences are flanked by *lox*P sites and propagated on Cre recombinase expressing 293 cells, yielded about a log less virus/ml than standard $E1^-/E3^-$-deleted vector, with only 0.01% helper contamination ***(26)***. It remains to be determined if these ''gutless'' Ad vectors improve transgene persistance in vivo.

2. Materials

2.1. Cloning, Purification, Propagation, and Titration of Adenoviral Vectors

1. Cell lines and media: $E1^+$ vectors are generally propagated on HeLa or KB cells, whereas first-generation Ad vectors ($E1^-/E3^-$ deleted) are most often grown on 293 monolayers. We routinely grow 293 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 m*M* L-glutamine (Gibco [Gaithersburg, MD]). Infection of cells with purified viral stocks are traditionally performed in DMEM with reduced serum level of 2%. For plaque assays, we use 2X MEM (Gibco [Gaithersburg, MD]) diluted 1 : 1 with 2X top agar.
2. 2X Top agar: 2% (w/v) bacterilogical agar (Difco), 0.2% (w/v) yeast extract, and 0.4% tryptone/100 mL H_2O and then autoclaved. This can be stored at 4°C for about 1 mo.
3. Sterile stock solutions: Phosphate-buffered saline (pH 7.2); 1*M* Tris–HCl (pH 7.4); 2*M* $MgCl_2$; glycerol (Gibco [Gaithersburg, MD]).
4. Sterile CsCl solutions: CsCl at 1.25 g/mL and 1.40 g/mL in 10 m*M* Tris–HCl (pH 7.4), 1 m*M* $MgCl_2$, and 1m*M* EDTA.
5. Viral suspension buffer (VSB): This solution containing 10% glycerol in 10 m*M* Tris (pH 7.4) and 1 m*M* $MgCl_2$ is used to dialyze the CsCl solution away from concentrated viral stocks to be used for in vivo experiments. A number of labs are currently using 10% glycerol–phosphate-buffered saline (PBS) with no adverse effects on the stability of high-titer stocks. There have been no controlled studies published that indicate the best buffer for long-term storage of Ad vectors.

2.2. Purification of Adenoviral DNA

1. 1*M* Tris–HCl (pH 7.4).
2. 1*M* EDTA
3. Proteinase K stock solution: Dissolve proteinase K at 20 mg/mL in 10 m*M* Tris–HCl (pH 7.4) and store at –20°C until needed. Proteinase K stock solution is thawed and diluted into buffer just prior to use for DNA extraction of concentrated virus (final concentration of 1 mg/mL proteinase K in 10 m*M* Tris–HCl [pH 7.4], 1 m*M* EDTA, 0.1% sodium dodecyl sulfate (SDS), and incubated at 37°C overnight).
4. Phenol–chloroform saturated with 10 m*M* Tris–HCl (pH 7.4) and 1 m*M* EDTA.
5. 100% ethanol stored at –20°C for DNA precipitations.

6. 3*M* sodium acetate (pH 5.2).
7. TE buffer: 10 m*M* Tris and 1 m*M* EDTA (pH 8.0), for resuspention of viral DNA.

2.3. DNA Transfections

1. 2X HEPES-buffered saline (HeBS): 5 g/500 mL HEPES (*N*-2-hydroxyethylpiperazine-*N* ' 2-ethanesulfonic acid), 8 g/500 mL NaCl, 0.37 g/500 mL KCl, 0.1g/500 mL, Na_2–HPO_4, 1 g/500 mL glucose at a final pH 7.05–7.10. The pH of the HeBS solution is critical for good precipitation of DNA and should be checked each time the solution is used. Sterilize the HeBS solution by filtration and store at 4°C in tightly closed container.
2. Carrier DNA. We use irrelevant plasmid precipitated with ethanol and resuspended in sterile H_2O at 1 mg/mL stock solution that is diluted to a final concentration of 10 μg/mL in HeBS.
3. 2 *M* $CaCl_2$ is made fresh each day in H_2O and filtration sterilized.

3. Methods

3.1. Viral Titration (Plaque Assay)

1. First-generation vectors are titered on 293 cells set up 1 d prior to use in six-well plates at approx 2×10^5 cells/well (75–80% confluent).
2. Dilutions of virus are performed the next day in DMEM containing 2% serum. All culture medium is removed and replaced with 1 mL of appropriate viral dilution to each well. Viral dilutions are adsorbed for 90 min in a 37°C humidified CO_2 incubator mixing every 30 min. Viral dilutions are removed and monolayers are overlaid with 4 mL/well of 1 : 1 mix of top agar and 2X MEM. The top agar is equilibrated at 42°C for 30 min before use and mixed with an equal volume of 2X MEM equilibrated at 30°C. The polymerization of overlay is rapid and must be accomplished quickly.
3. Incubate overlays in a 37°C humidified CO_2 incubator. Plaques will appear in 5–7 d and should be counted between d 10 and 14.

3.2. Viral Propagation

1. We generally use (80–100) 500-cm^2 tissue culture flasks (nunc) that at approx 90% confluency have approx 3×10^7 293 cells/flask. We routinely infect the cells at 5–10 PFU/cell. Under these conditions most first-generation vectors will display complete cytopathic effect (CPE) by 36–44 h postinfection.
2. Virally laden cells are collected and concentrated by low-speed centrifugation and resuspended in 0.5–0.75 mL of supernatant/flask.
3. Infected cells are disrupted by repeated rounds of freezing and thawing.
4. This crude viral lysate (CVL) is clarified by centrifugation at 15,000*g* for 20 min and 10% glycerol added to generate a relatively concentrated stock of *(1–5)* $\times 10^9$ PFU/mL and stored at –70°C.

3.3. Purification of Adenovirus Vectors

1. Clarified CVL can be highly purified by banding the Ad vector on a two-tier CsCl gradient of 1.25 g/mL and 1.40 g/mL in 10 m*M* Tris–HCl (pH 7.4) and 1 m*M* $MgCl_2$ and spinning in a SW 41 rotor at 150,000*g* at 20°C for 1 h.

2. Banded virus is pooled and subjected to an isopycnic CsCl gradient (1.33 g/mL) in a SW 41 rotor at 150,000*g* at 20°C for 16 h.
3. The final viral bands are collected in as small a volume as possible and are then dialyzed extensively (four changes with at least 1000 volumes for at least 1 h/change) against VSB at 4°C to remove CsCl.

3.4. Purification of Adenovirus DNA

1. Dialyzed banded virus is suspended in 1mg/mL proteinase K in 10 m*M* Tris–HCl (pH 7.4), 1 m*M* EDTA, 0.1% SDS, and incubated at 37°C overnight.
2. Viral DNA is then extracted with phenol–chloroform saturated with 10 m*M* Tris–HCl (pH 7.4) and 1 m*M* EDTA.
3. Viral DNA is then precipitated using 1/10 volumes 3*M* sodium acetate and 2 volumes 100% ethanol and resuspended in TE and appropriate restriction buffer for restriction digestion.

3.5. Generation of Adenoviral Vectors by In Vivo Homologous Recombination

1. The condition of the recipient 293 cells is the most important variable in the cotransfection process. The 293 cells should be as early a passage as possible and at a confluency of approx 70% in a 60-mm dish.
2. Place 350 µL of 2X HeBS and 350 µL of sterile H_2O in a sterile tube (Falcon 2058) with 10 µg of carrier DNA.
3. Add 10 µg of shuttle plasmid containing the transgene and add 10 µg of vector plasmid in 50 µL H_2O to the tube in **step 2**.
4. Add 50 µL of 2*M* $CaCl_2$ and use a single wrist snap to mix vigorously and let incubate at room temperature for 30 min.
5. Rinse 60-mm dish with 3 mL 1X HeBS and leave 0.5 mL of HeBS on dish. Add contents of tube after incubation and let dish stand at room temperature for 20 min.
6. Add 2.5 mL DMEM with 2% FCS and incubate at 37°C in 5% CO_2 for 18 h.
7. Overlay monolayer with top agar and pick single plaques (d 7–14) to amplify and screen for transgene. Initial screening can be accomplished using Polymerase chain reactions, enzyme-linked immunosorbent assay, immunoprecipitation, or Western blotting but should be confirmed using a functional bioassay before large-scale production of the Ad vector.

4. Notes

1. The cloning of adenoviral vectors has become standard practice in a number of labs with each having a choice method that is most effective for their purposes. In our hands, homologous recombination is more than adequate for cloning most transgenes into adenoviral vectors. The condition of the recipient 293 cells is the most important variable in the homologous recombination process. The 293 cells should be as early a passage as possible and at a confluency of approx 70% in a 60-mm dish. Additionally it is best that the cell never become confluent and are dividing rapidly. At times, we expose our transfections to 3–5 s of ultraviolet light to upregulate

the frequency of homologous recombination. Careful calibration of the light source is essential to avoid killing the 293 cells (*see* **Subheading 3.5.**).

2. The pH of the HeBS solution is critical for good precipitation of DNA and should be checked each time the solution is used. When the HeBS solution is pH 7.05, an extremely fine ppt. that may require overnight incubation for the cells to take up enough complexes. One may speed the complexation procees by placing the cells in an incubator at 5% CO_2 or slow the complexation by incubating in 10% CO_2. The cells should be observed every 2 h to prevent too many complexes (>70/cell) from entering the cell or significant toxicity may be observed. The transfected cells can be shocked with 15% glycerol/DMEM or 25%DMSO/DMEM to increase the amount of complexes delivered in the cell, but caution must be exercised. Shocking with the 15% glycerol/DMEM for more than 80 s results in exponential cell death. Likewise treatment with 25%DMSO/DMEM for more than 180 s also results in exponential cell death. Overlayering the cells after shocking should not be attempted without allowing the cells to recover overnight (*see* **Subheading 3.5.**).
3. The shuttle and viral plasmids should both be grown in bacteria strains that are stable. We use Stbl2 cells (Gibco [Gaithersburg, MD]) to avoid rearrangement of the viral plasmid pJM17 ***(12)***. We inoculate 50 mL of superbroth (supplemented with 100 µg/mL ampicillin) for overnight culture at 30°C with a colony or 50 µL of glycerol stock of pJM17. The overnight culture is then used to inoculate 500 mL of superbroth (supplemented with 100 µg/mL ampicillin) and allowed to grow 4 h at 30°C and a finally 2 h at 37°C. Plasmids are harvested by standard alkaline lysis and highly purified using cesium trifluoroacetate (Pharmacia [Piscataway, NJ]). Care must be exercised in processing the pJM17 plasmid to avoid damaging the viral backbone (*see* **Subheading 3.5.**).
4. Problems with the plaque assay are not uncommon if the top agar and 2X MEM are not properly equilibrated at 42°C and 30°C, respectively. Alternatively, low-melting agar can be used for isolation of plaques, but polymerization of overlay is slow and extra time should be allowed for complete polymerization of the overlay before returning them to the incubator (*see* **Subheadings 3.2.** and **3.5.**).
5. The cloning of toxic transgenes into adenoviral vector can be problematic. We often clone such constructs in reverse orientation to ablate the effects of the E1A enhancer. In some cases, it is necessary to place the transcription unit under the control of an inducible/repressible promoter so that vector can be cloned (*see* **Subheading 3.5.**).

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14

Electron Microscopic Assessment of Adenovirus-Mediated Transfer

Catherine E. Sarraf

1. Introduction

This chapter describes the method of preparing and observing hepatocellular carcinoma (HCC) and surrounding normal liver cells infected with therapeutically *p53*-transfected adenovirus (Ad-*p53*), so that morphology of the cells and viruses, and crucially their relationships to each other, are revealed. In standard practice, ultrastructural analysis of viruses carried in body fluids (e.g., stool or mucus) is sufficient for diagnosis, using the technique of phosphotungstate — dark field staining — of the aqueous extract. That method, however, is not suitable when one needs to examine precise subcellular location of viruses *in situ*, with tissue and cells intact, for complete pathological assessment; here, we describe our method ***(1)*** for transmission electron microscopy of the ultrastructure of virus-infected tumors. Tissue fixation, osmication, embedding, section cutting, and observation of Ad-*p53* infection will be included.

1.1. Electron Microscopy

In microscopy, it is important to note the difference between magnification and resolution of an image. Electron microscopy relies heavily on photographic and electronic (television, video) images to record information obtained by direct observation. Any photograph can be magnified to any size; the result is a simply a larger photograph displaying information no more detailed than the original. In contrast, when a high-resolution image is magnified, information (that had previously been too small) becomes visible. Resolution is defined as the distance that can be identified by an instrument,

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

which separates two adjacent structures — the smaller the distance, the better the resolving power. Electrons, produced in the cathode of the electron microscope (EM), are high-energy, negatively charged particles and their wavelike properties plus their propagation across the evacuated column of the microscope are both conducive to the production of high-resolution images.

Very thin sections of the specimen need to be cut and placed in the path of the electron beam of the transmission EM (TEM). Electrons pass through the sample to the extent that they are able, depending on their energy (the voltage at which they were produced): straight through ''electron-lucent'' structures, stopped by ''electron dense,'' high-atomic-number structures, and transmitted to varying degrees through others. The emerging beam impinges on a fluorescent screen and/or photographic plate, and the variegated pattern produced is an image of the structures through which the beam has passed. Because the electrons initially all have the same energy (there is no spectrum of equivalent wavelengths), the image has no color; it ranges from white through the shades of gray to black. Computer-assisted imaging can assign false colors to selected structures, but this is aesthetic, not a basic property of the system.

1.2. Ultrastructure of Adenovirus-Infected Lesions

Electron microscopic assessment of adenovirus-mediated transfer to liver lesions includes evaluating the status of surrounding normal liver cells as well as that of tumor cells. Hepatocytes and HCC cells are comparatively large, usually greater than 20 μm, and each contains its complement of subcellular organelles. Clearly, to benefit from TEM, one has to be able to identify cells, organelles, and viruses; adenoviruses, depending on the exact serotype, vary in size between 70 and 90 nm (0.07 and 0.09 μm). High-quality, modern electron microscopes, working in optimal conditions, routinely resolve details of 1 nm or less; therefore, in tissue sections infected with adenovirus – cellular characteristics, morphology of viral coats and central cores are all easily recognized; viruses may be sequestered and enzymatically degraded in cytoplasmic vacuoles **(Fig. 1)**. In the case of HCC, great care must be taken when identifying the presence of therapeutic adenovirus, as the development of HCC may be associated with prior infection, commonly with hepatitis B virus (HBV) or hepatitis C virus (HCV); immunolabeling of appropriate viral antibodies at the EM level provides specific identification ***(2)***.

When Ad-*p53* has been administered to patients with HCC or to animals in model investigations ***(3)***, two distinct strategies have been used: introducing viruses into the afferent blood supply (hepatic artery or hepatic portal vein) or directly into the tumor. In the former, viruses can be found within sinusoids/tumor vasculature, which they must leave before they can enter cells of the lesion; when adenovirus has been applied directly to the tumor, their first

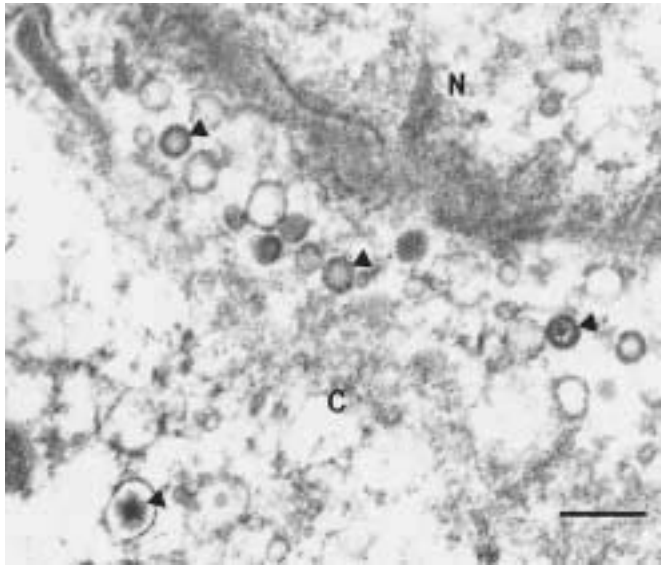

Fig. 1. High-power electron micrograph showing numerous adenoviruses (arrows) inside a hepatocellular carcinoma cell; N is the cell nucleus and C is the cytoplasm. Viruses gradually lose their integrity in the cytoplasmic environment, and different stages are visible: both coat and core intact, coat only, and degradation inside a vacuole. Bar = 0.2 μm.

obstacle is the plasma membrane of the target cell. Our group has documented adenoviruses between vascular endothelial cells and within stromal and tumor cells, after the appropriate means of administration. Adenovirus burden of any specific cell depends on the concentration of the original dose, time elapsed since the infection, and whether viruses have deteriorated in the interim ***(4)***. Once inside target cells, adenoviruses proceed to the vicinity of the nucleus, which they circumscribe and cause to shrink **(Fig. 2)**. The nuclear membrane becomes disrupted, and although adenoviruses have not been seen entering uninjured nuclei, they attack the membrane and come to occupy space that had been intranuclear **(Fig. 3)**. Ultimately, the result is cell death by apoptosis, whose morphological features — chromatin condensation with nuclear and cytoplasmic shrinkage — are well illustrated by transmission electron microscopy ***(5)***.

2. Materials

2.1. Glutaraldehyde

Stock EM grade glutaraldehyde is 25% histochemically pure; freshly mix 1 mL of this with 11.5 mL of phosphate buffer pH 7.2, per procedure.

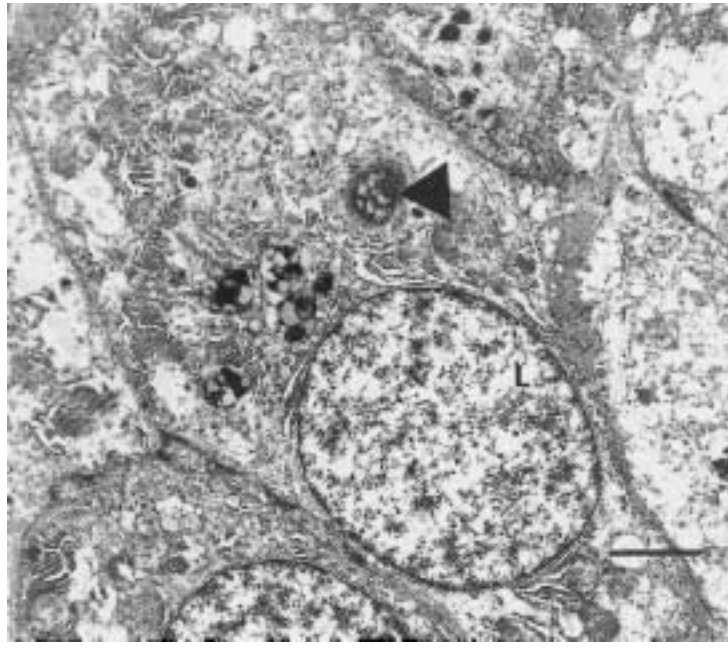

Fig. 2. Low-power electron micrograph of a binucleate HCC cell. One nucleus (L), is large and rounded, but the second (arrow), under viral attack, has shrunken. Bar = 2.5 µm.

2.2. Phosphate Buffer

Solution I: $NaH_2PO_4 2H_2O$, 15.6 g in 500 mL distilled H_2O 0.2 *M* (acid).
Solution II: $Na_2HPO_4 2H_2O$, 17.8 g in 500 mL distilled H_20 0.2 *M* (alkaline).
Buffer mixture, as follows to 0.1 *M:*

Solution I	70 mL
Solution II	180 mL
Distilled H_2O	250 mL

Adjust to pH 7.2 with solutions I and II.

2.3. Osmium Tetroxide (Johnson Mathy, Royston, Cambs, UK)

Note: This vapor is hazardous.
Standard Millonig's method ***(6)***; always prepared inside a fume hood.

Solution I	100 mL 2.6% $NaH_2PO_4 2H_2O$ in distilled H_2O
Solution II	12.6 mL carbonate-free NNaOH in 7.4 mL distilled H_2O
Solution III	0.54 g (= 5.4%) glucose in 10 mL distilled H_2O
Solution IV	83 mL Solution I + 17 mL Solution II, adjusted to pH 7.2

Combine the following, inside a dark hood and leave overnight to dissolve:
Osmium tetroxide 1 g ampoule, to be broken

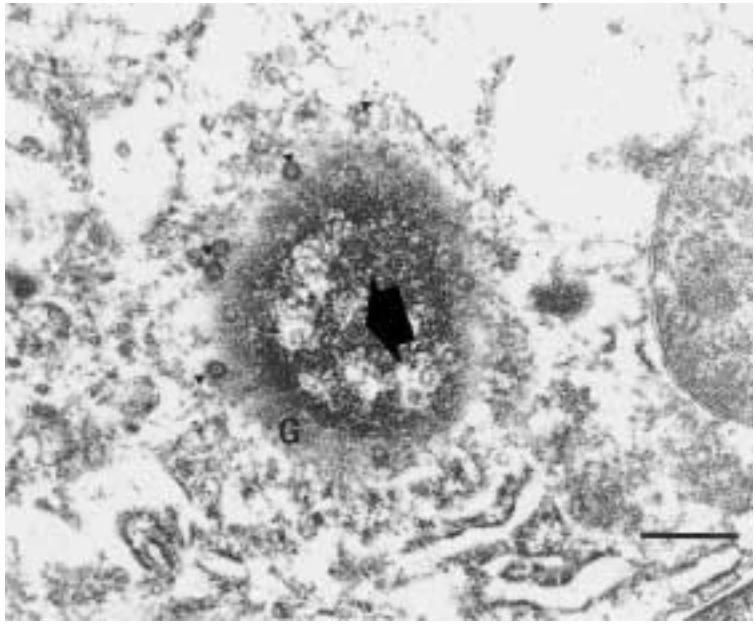

Fig. 3. High-power electron micrograph of the smaller nucleus shown in **Fig. 2**, which has several associated viruses (small arrows); the nuclear membrane has been disrupted and replaced by a granular penumbra (G), and chromatin has undergone some condensation (large arrow). This is the typical morphology of Ad-*p53*-attacked nuclei. Bar = 0.5 µm.

Solution III 10 mL
Solution IV 90 mL

The final solution should be pale straw colored and precipitate free. Store in a tightly closed dark bottle at 4°C; discard when solution darkens.

2.4. Acidified Dimethoxypropane (DMP)

TAAB Laboratories Equipment Ltd., Aldermaston, Berks, UK.
(TAAB is a registered trade -mark)
2,2 DMP 100 mL
HCl (conc.) 0.05mL (this is 1 drop)

2.5. Alcohols

Absolute alcohol (AA) and sequential dilutions of AA in distilled water should be used for dehydration, rising from low dilution to AA.

2.6. Epoxy Resin

For morphology, a mixture of TAAB polymers is used. Blocks of suitable rigidity polymerize from the following mixture:

TAAB resin 50 mL
DDSA hardener 45 mL dodecenylsuccinic anhydride 964
DMAMP accelerator 5 mL 2,4,6-tri(dimethylaminomethyl) phenol

2.7. Toluidine Blue

Toluidine blue 1 g
Sodium tetraborate 100 g

2.8. Methanolic Uranyl Acetate (Saturated)

Note: Uranyl acetate is radioactive and is stored in lead cylinders.
Methanol 100 mL
Powdered uranyl acetate >100 g
Prepare a saturated solution by adding a surfeit of powdered uranyl acetate until it will no longer dissolve and a minor precipitate remains; the required volume is filtered each time, immediately before use.

2.9. Lead Citrate

Standard Reynold's method *(7)*
Lead nitrate 1.33 g $PbNO_3$ in 15 mL distilled H_2O
Sodium citrate 1.76 g $Na_3(C_6H_50_7)2H_2O$

Mix until a precipitate forms: dissolve this drop by drop with addition of sodium hydroxide.

Sodium hydroxide 8 mL NNaOH (carbonate free) in 50 mL distilled H_2O
Filter before use.

3. Methods

For EM, fresh tissues need to be prefixed in glutaraldehyde, then postfixed in an osmium salt to prevent metabolic deterioration. Blocks are then dehydrated, as water and epoxy resin (the next stage) are immiscible; samples are impregnated with the liquid stage, which is then polymerized. Blocks need to be embedded in resin for rigidity, to allow them to be thinly sliced and to withstand the rigors (vacuum, electron bombardment) of the environment inside the EM. First, a ''thick'' (1μm) section is cut from the block, collected on a glass slide, and examined at the light microscope level; this is most often stained with Toluidine Blue. Viruses are not visible at the light microscope level, but they frequently distort cell contents in characteristic ways, which may indicate their presence. The 1-μm sections of many blocks can be scanned rapidly to determined whether they contain such morphological deformation, and if so, an ultrathin section is cut from the same block. Ultrathin sections, approximately 100 nm thick, are collected on metal grids, then counterstained with uranyl acetate and lead citrate. They are inserted into the EM column and

furnish images of the same structures seen in the ''thick'' section, but with higher resolution and magnification.

1. Remove the tissue sample from the body and directly immerse it in 2% glutaraldehyde.
2. Quickly — by hand, with a sharp blade — slice the sample into blocks of not more than 1 mm^3, not allowing them to dry. Reimmerse the small blocks in fresh 2% glutaraldehyde and leave to fix for at least 1–2 h at 4°C, rotating if possible (*see* **Note 1.**).
3. Rinse blocks in phosphate buffer, pH 7.2, then store in this solution until it is convenient to continue processing (days to weeks, if necessary).
4. Postfix in 1% Millonig's phosphate-buffered osmium tetroxide, 1 h, 4°C with rotation (*see* **Note 2.**).
5. Rinse well in distilled water 2 – 15 min with rotation.
6. Dehydrate, 2X (7–15 min) in acidified 2,2 dimethoxypropane (DMP) or, alternatively, dehydration can be achieved by passing the tissue through graded alcohols; in that case, 30 min each, at room temperature (RT): (percentages of ethanol) 30%, 50%, 70%, 90%, 100%, repeat 100%.
7. To infiltrate the tissue with resin, immerse blocks in the combined liquid TAAB resin, 1 h with rotation. Change resin twice and repeat.
8. Embed in fresh resin in appropriate molds, then polymerize in oven at 60°C, overnight.
9. Remove the blocks, allow them to cool, and eject them from their molds.
10. Trim blocks, then cut 1-µm sections on the ultramicrotome, with a glass knife; collect the sections on glass slides.
11. Stain 1-µm sections with toluidine blue in 1% sodium tetraborate, for 3–4 min at 50°C on a hot plate. Mount in synthetic mountant and view under the light microscope (*see* **Note 3.**).
12. Blocks may be further trimmed to retain a chosen zone only; it is easier to cut ultrathin sections of smaller areas. Ultrathin sections ~ 100nm thick are cut on the ultramicrotome with a diamond knife, spread with chloroform and are collected on nickel or copper grids.
13. Counterstain grid-mounted sections with first uranyl acetate, then lead citrate (*see* **Note 4.**).

    A. Uranyl acetate counterstaining: Make 1 drop of methanolic uranyl acetate for each grid, on a clean sheet of dental wax. Float each grid on a drop, section side down, and cover the whole assemblage to limit exposure to the air. Leave for 2–3 min. Jet wash each grid individually in double-distilled water.

    B. Lead citrate counterstaining: Make 1 drop of Millipore-filtered Reynolds lead citrate for each grid, on a clean sheet of dental wax. Float each grid on one drop, section side down, and cover the whole assemblage to limit exposure to the air. Leave for 10 min.
14. Jet wash each grid individually in double-distilled water and lay on fiber-free filter paper to dry for 2–3 min.
15. Observe on the electron microscope, usually operated at 60–80 kV.

4. Notes

1. It is essential to proceed immediately from **step 1** to **step 2**, as glutaraldehyde has poor powers of penetration; until the tissue is cut into small blocks, central regions remain unpreserved. Liver has the reputation of being difficult to fix; our group has no trouble, so long as the blocks are small enough.
2. Postfixation in osmium tetroxide also imparts electron density to many organelles such as cytomembranes, nuclei, and mitochondria. Blocks turn black.
3. At this stage, the 1-µm sections of many blocks can be rapidly examined; blocks which show virus-infected cells can be pursued at the electron microscope level.
4. Lead citrate readily reacts with carbon dioxide from the air, forming a black precipitate of lead carbonate which contaminates the preparation. To prevent this, the stock solution must be tightly stoppered, stored at 4°C, and discarded if there is the slightest hint of cloudiness. During staining, one or two pellets of sodium hydroxide are commonly placed near the floating grids, not touching, but inside the cover. These absorb surrounding gaseous carbon dioxide. Uranium salts react preferentially with proteins and lead salts preferentially with lipids. Using both visualizes subcellular organelles and lipid bilayer membranes in the preparation.

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15

p53 Plasmid Preparation and Techniques for Analysis of Gene Transfer and Expression

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer death worldwide and is especially prevalent in certain areas of Africa and Asia. The most important etiological factor is infection with the hepatitis B or C virus. Treatment is generally unsatisfactory as the majority of patients are not suitable for surgical resection and chemotherapy is not particularly effective.

Normal wild-type p53 acts as a suppressor of cell proliferation after DNA damage and it has been shown that over half of HCCs have mutant p53 ***(1)***. Restoration of p53 activity in vitro in HCC cell lines that have null or mutant p53 induces apoptosis ***(2)*** and in vivo results have shown tumor growth inhibition in animal models of HCC ***(3)***. In addition, many anticancer agents, including chemotherapeutic drugs, mediate their effects by inducing p53-dependent apoptosis ***(4)***. A theoretical problem with clinical use of p53 is the potential for toxicity in normal tissues from overexpression of the exogenous gene. To our knowledge there is only one report of this that found dose-dependent p53 toxicity in rat neurons ***(5)***.

Adverse effects from vector toxicity remain a cause for concern. Although some of the earliest work was done using retroviruses, problems with this vector include transfection of dividing cells only and integration into the host genome, causing both tumor and nontumor tissue to be permanently modified ***(6)***. Adenoviral and plasmid DNA vectors (pDNA) have become more popular because they do not integrate into the host genome ***(6,7)***. A theoretical risk with the use of pDNA is the generation of an

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

autoimmune disorder; however; there is no evidence that this actually occurs ***(7)***. Adenoviral vectors have been shown to cause some hepatic inflammation and dysfunction when given in high doses to experimental animals ***(6)***.

A problem with the methods currently available for gene delivery is poor transduction efficiency. In an attempt to overcome this, direct intratumoral injection and locoregional infusion (hepatic artery) have been used to increase the efficiency of gene transfer to tumor tissue while minimizing the exposure of normal tissue.

The following sections describe the techniques used in our laboratory for the preparation of p53 plasmid and analysis of tumor tissue for gene transfer and expression.

2. Materials

1. Agar (Difco Laboratories, Detroit, MI).
2. Luria broth (LB) base (Miller's LB broth base) (Sigma, Dorset, UK): dissolve 25 g in 1 L of deionized water. Make large amounts and aliquot in 500-mL and 1000-mL bottles. Sterilize by autoclaving and store at 4°C.
3. Ampicillin (Sigma): sodium salt, dissolve 50 mg/mL in sterile deionized H_2O. Filter the solution twice through 0.2-µm filters in a Class II laminar flow cabinet. Aliquot the solution in 1-mL and 10-mL sterile tubes and store at –20°C. Working concentration should be 50 µg/mL.
4. Plasmid: pC53-SN3, encodes human wild-type p53 under the control of the cytomegalovirus (CMV) promoter and has the antibiotic resistance marker genes, ampicillin, and neomycin. (Kindly provided by Dr. Bert Vogelstein.)
5. TE buffer: 10 m*M* Tris–HCl, pH 8.0 and 1 m*M* EDTA. Sterilize by autoclaving and store at room temperature.
6. *Escherichia coli*: XL-1 Blue (Stratagene, Amsterdam, The Netherlands).
7. Glycerol (BDH Ltd., Dorset, UK): sterilize by autoclaving.
8. Qiagen Plasmid Mega, Giga kits (Qiagen Ltd., West Sussex, UK).
9. Absolute ethanol (BDH Ltd.).
10. 3*M* sodium acetate (Sigma): anhydrous. Adjust pH to 7.4. Sterilize by autoclaving.
11. 70% ethanol: diluted in sterile deionized H_2O.
12. Saline: sodium chloride for intravenous use, 0.9% w/v.
13. Lysis buffer: 0.32*M* sucrose, 10 m*M* Tris–HCl, pH7.5, 5 m*M* $MgCl_2$, 1% (w/v) Triton X-100.
14. 10% sodium dodecyl sulfate (SDS) in double-distilled H_2O (ddH_2O).
15. Proteinase K solution: proteinase K dissolved in autoclaved 0.075*M* NaCl, 0.024*M* EDTA, pH 7.5, at a concentration of 10 mg/mL.
16. Phenol–Chloroform mixture: 1 : 1 ready mixed (purchased from Sigma–Aldrich). This mixture is Toxic and should be handled with gloves in a fume hood.
17. Choloroform.
18. DNA *Taq* polymerase enzyme.
19. KCl, 10X *Taq* polymerase buffer (supplied with the DNA *Taq* polymerase enzyme).
20. Deoxnucleotide triphosphates mixture (dNTPs): 1.25 m*M* dATP, 1.25 m*M* dCTP, 1.25 m*M* dGTP, 1.25 m*M* dTTP.

21. Loading buffer: 40% sucrose, 0.025% w/v Bromophenol Blue, 0.025% w/v xylene cyanol.
22. Ethidium bromide (EtBr) solution: 10 mg per mL, dissolved in distilled H_2O. Highly Toxic; handle with care. Store in a dark bottle at 4°C. Disposing EtBr and cleaning contaminated/spillage areas are carried out according to the regulations and rules set by the senior staff in charge of the laboratory.
23. 10X TBE running buffer: 890 m*M* Tris–HCl, pH 8.0, 890 m*M* boric acid, 200 m*M* EDTA (disodium salt).
24. 5X SDS/electrophoresis buffer: Tris base 15.1 g, glycine 94 g, SDS 5.0 g, add H2O to 1000 mL, store at 4°C.
25. 1X SDS/sample buffer: 50 m*M* Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 0.1% Bromophenol Blue; store at room temperature. Add 10 μL β-mercaptoethanol to 1 mL 1X SDS/sample buffer just before use.
26. 10X TBST: 87.7 g sodium chloride, 24.2 g Tris base, 800 mL H2O, adjust pH to 7.5. Add Tween 10 mL and H2O to 1000 mL.
27. Transfer buffer: 1.5 g Tris base, 400 mL H2O, 100 mL methanol.
28. Blocking buffer: Bovine serum albumin (BSA) 1.5 g, 1X TBST 100 mL, Make fresh and keep in 4°C.
29. Acryamid/bisacrylamid (AB) 30 : 0.8% Anachem Ltd.
30. 1.5*M* Tris–HCl pH 8.8.
31. 1.0 *M* Tris–HCl pH6.8.
32. ECLTM Western Blotting Detection Reagent 1, ECLTM Western Blotting Detection Reagent 2 (Amersham Life Science).
33. Primary antibodies: Mouse anti-human p53 (PAb 1801) (SeroTEC) recognizes both wild-type and mutant p53 protein, use as 1 : 2000 dilute. Mouse anti-human p53 (PAb 240) (SeroTEC) recognizes mutant p53 protein, 1 : 2000 dilution. Mouse anti-human p21 (187) (Santa Cruz Biotechnology, Inc.), 1 : 1000 dilution. Goat anti-human Bax (P-19)-G (Santa Cruz Biotechnology, Inc.), 1 : 2000 dilution. Goat anti-human Lamin B (C-20) (Santa Cruz Biotechnology, Inc.), 1 : 2000 dilution.
34. Secondary antibodies: Anti-mouse IgG-HRP (Santa Cruz Biotechnology, Inc.), 1 : 2000 dilution. Anti-goat IgG-HRP (Santa Cruz Biotechnology, Inc.), 1 : 2000 dilution.
35. Trans-Blot® SD, Semi-Dry Transfer Cell (Bio-Rad).
36. HybondTM ECLTM, nitrocellulose membrane (Amersham Life Science).
37. Soniprep 150, Sanyo.
38. Kodak X-OMAT Scientific Imaging Film (Sigma).
39. Power supply (Bio-Rad).
40. Protean II 16-cm cell (Bio-Rad).
41. 10X 3-[*N*-Morpholino] propanesulfonic acid (MOPS) running buffer: MOPS 20.6 g, sodium acetate 3.28 g, 0.5*M* EDTA 10 mL, 400 mL, adjust pH to 7.0, add H2O to 500 mL. Autoclave and store in the dark.
42. Loading dye: 1 m*M* EDTA pH 8.0, 50% glycerol, 0.25% Bromphenol Blue, 0.25% xylene cyanol, H2O to 20 mL; Autoclave.
43. 20X SSC: 3*M* sodium chloride (175 g/L), 0.3 *M* sodium citrate (88 g/L), adjust pH to 7.0 with 1 *M* HCl.
44. Stripping buffer: 0.1X SSC, 1% SDS, 40 mM Tris–HCl pH 7.5–7.8.

45. Diethylpyrocarbonate (DEPC) treatment of solution: Add 0.2 mL DEPC to 100 mL of solution to be treated. Shake vigorously to get the DEPC into solution. Autoclave the solution to inactivate the remaining DEPC. DEPC is a suspected carcinogen; handle with care.
46. Hybridizer 700 (Stratagene).
47. Stratalinker 1800 (Stratagene).
48. HYPER-PRIMER Quick kit (Bioline).
49. Rapid-Hyb buffer (Amersham).

3. Methods

3.1. Plasmid Preparation

3.1.1. Stock LB-Agar Plates Preparation

The following procedure should be carried out near a Bunsen burner (*see* **Notes 1** and **2**).

1. Add 1% of agar to LB. Prepare enough mixture for about four Petri dishes.
2. Sterilize by autoclaving and allow LB–agar to cool to approximately 45°C.
3. Add 1 µL/mL of ampicillin (*see* **Note 3**).
4. Pour approximately 25 mL into each Petri dish and allow to set at room temperature.
5. Incubate dishes at 37°C overnight to ensure that there is no microbial contamination.

3.1.2. Transformation of E.coli

1. Defrost *E. coli* and aliquot approximately 50 µL into each sterile microfuge tube.
2. Transfer a small volume (1–2 µL) of plasmid solution onto bacterial aliquot (*see* **Note 4**).
3. Mix by gentle tapping followed by a brief spin.
4. Incubate on ice for 30 min.
5. Spread the batreria onto the LB–agar plate using a sterile spreader.
6. Incubate at 37°C overnight.

3.1.3. Glycerol Stock Preparation

1. Prepare "mini" cultures. Place 5 mL of LB in a sterile universal tube and add 5 µL of ampicillin stock solution.
2. Transfer one colony of transformed *E. coli* from the LB–agar plate into the tube using a sterile loop near a Bunsen burner (*see* **Note 5**).
3. Incubate in an incubator shaker at 37°C overnight.
4. (The next day) Transfer 1.5 mL of the culture into a sterile cryo-tube (Gibco-BRL, Paisley, UK) and add 0.3 mL of glycerol (*see* **Note 6**).
5. Mix by inversion about six to eight times and store at –80°C.

3.1.4. Plasmid Extraction and Purification

Extraction/purification kits are commercially available (e.g., Qiagen Plasmid Mega, Giga Kits purchased from Qiagen Ltd., West Sussex, UK). The plasmid is extracted according to the manufacture's protocol.

1. In sterile universal tubes, place 10 mL LB broth per tube, then add 10 µL ampicillin stock solution. Using a sterile loop, transfer a ''pinch'' of the bacterial glycerol stock into each of the tubes. Recap tubes and mix contents by vigorous shaking, then place in a shaking incubator overnight, at 37°C. The contents of each tube will be used to seed 250–500 mL LB broth.
2. The following day, use 2-L sterile conical glass flasks to prepare large cultures. The number of flasks used will depend on the amount of plasmid to be prepared and the kit used. Use 500 mL LB broth per flask. Add 500 µL ampicillin stock solution, followed by a single 10-mL culture from **step 1**. Recap the flasks and incubate flasks in a shaking incubator (200–250 throws/ min) overnight, at 37°C.
3. Extract the plasmid using the kit and according to the manufacturer's instructions.
4. Precipitate plasmid DNA in sterile tubes (e.g., 50-mL Falcon tubes).
5. Centrifuge tubes at 1700*g* for 20 min at 4°C.
6. Discard the supernatant and resuspend pellet in approximately 1 mL of 70% ethanol.
7. Transfer the suspension in sterile microfuge tubes.
8. Centrifuge tubes at 10,000*g* at room temperature for 2 min and discard supernatant.
9. Spin down briefly (approx 2 s) and remove residual solution.
10. Place the tubes into a heating block at 55°C for approximately 1 min with lids open in order to evaporate residual ethanol.
11. Let tubes stand at room temperature for 2 min.
12. Add 0.5–1.0 mL of TE buffer, place tubes at 4°C, and allow plasmid DNA to dissolve.

3.1.5. Sterilization of Extracted Plasmid

Determine the concentration of the dissolved plasmid and sterilize the final solution as follows:

1. Use a spectrophotometer set for ultraviolet (UV) absorption at 260 nm. Dilute 10 µL of concentrated plasmid in TE buffer to a total volume of 1 mL. A reading of 1 corresponds to 50 µg of DNA (plasmid or genomic). Work out the original concentration of the undiluted solution.
2. In a Class II laminar flow hood, dilute the plasmid using TE buffer to give the final concentration required for your protocol. Filter the final solution twice through a sterile 0.2-µm filter in a sterile universal tube using a sterile disposable syringe. Aliquot the sterilized solution in sterile cryo-tubes and store at 4°C until use.
3. Samples of the sterilized plasmid should be tested for bacterial, viral, and mycoplasma contamination.

3.2. Detection of Gene Transfer and Expression in Treated Tissues

Tumor biopsies should be snap-frozen in liquid nitrogen. Use cryo-tubes to store biopsies and process samples immediately or store at –80°C.

3.2.1. DNA Extraction

1. Crush the frozen biopsy inside the cryo-tube using a sterile glass rod.
2. Add 0.5 mL lysis buffer (containing 0.5% SDS/mL), and add 100 µg proteinase

K/mL and recap the tubes. Place tubes in a shaking incubator (gentle shaking, about 70–80 throws per minute) at 37°C overnight.
3. Transfer digested sample into a fresh sterile 1.5-mL microfuge tube, and add an equal volume of phenol–chloroform and 1/10 volume NaAc. Mix contents using a roller or gyratory shaker for 15 min, at RT.
4. Centrifuge tubes at 7000*g* and RT for 10 min; then, carefully, without disturbing interface layer, transfer the top aqueous layer into a fresh microfuge tube.
5. Add an equal volume of phenol–chloroform and mix on a shaker for 15 min at RT.
6. Repeat **steps 5** and **6** until no interface layer is visible, then, transfer the aqueous layer into a fresh microfuge tube.
7. Precipitate DNA by adding an equal volume of ice-cold absolute alcohol and mix by inversion (about 10 times), then place in –20°C freezer overnight. At this point tubes can be stored in freezer for several months. If the DNA samples are going to be analyzed the same day, then place the tubes on dry ice for 1 h.
8. If DNA is required, then centrifuge tubes at 7000*g* and 4°C for 10 min. Discard the supernatant and resuspend the DNA pellet in 0.5 mL 70% alcohol.
9. Transfer the suspension into a sterile 1.5-mL microfuge tube and centrifuge at 7000*g* and RT for 10 min.
10. Discard the supernatant and let pellets air-dry for 2–3 min; then, resuspend each pellet in 0.5 mL TE. Place tubes in fridge to allow DNA to dissolve slowly.

3.2.2. Restriction Enzyme Digestion of DNA

1. Place approx 5 µg DNA sample (plasmid DNA, genomic DNA, and polymerase chain reaction [PCR] product) in a microfuge, then add 4 µL 10X buffer (supplied with enzyme) and 20 units restriction enzyme. Add ddH_2O to make up the total volume to 40 µL; then, mix contents by gentle tapping.
2. Centrifuge tube for 1 s (pulse centrifugation). Place the tubes for about 4 h at the appropriate optimal temperature for the enzyme used. Sometimes, a longer period of incubation is required. In fact, to ensure that digestion is complete, the tubes could be incubated overnight.
3. Analyze the undigested and digested DNA samples alongside a DNA marker ladder using agarose gel electrophoresis (a 1% agarose gel and add 0.5 µL of EtBr per 10 mL of prepared gel.).

3.2.3. Detection of Gene Transfer Using PCR – neo^r as a Marker Gene

Tumor biopsies can be analyzed for exogenous genes transfer by using for example, the neomycin resistance gene as a marker gene. Ensure that all disposable tips, tubes and plasticware, and solutions used are sterile. Use fresh tips when pipetting the various solutions and samples. The following protocol is used with a thermo-cycler (PCR machine) that does not require mineral oil to be placed on top of the reaction mixture. If mineral oil is required, then carefully and gently place a drop of the oil on the surface of reaction mixture in each tube.

1. Dilute each DNA sample in sterile ddH_2O to a final concentration of approx 25

ng/μL. Place a 10-μL sample in the reaction tube.

2. In a sterile 1.5-mL microfuge tube, prepare sufficient ''master mix'' solution for the samples to be analyzed. Per sample: 5 μL 10X reaction buffer, 8 μL dNTPs mixture, 1 μL primer 1, 1 μL primer 2, approx 24.5 μL sterile ddH_2O, approx 2 units *Taq* polymerase (approx 0.5 μL, depending on the concentration of the polymerase). Mix contents of tube by gentle tapping, followed by brief centrifugation.
3. Add 40 μL ''master mix'' to each DNA sample. A negative control tube can be included in which 10 μL sterile ddH_2O are used instead of DNA. Gently triturate samples (mixing sample by up/down pipetting). Cap the tubes and place in PCR machine and start the run. For example, to detect a 791-bp fragment of *neo*^{r}, the following primers ***(8)*** and PCR cycles could be used:

primer 1 (forward):		5' CAA GAT GGA TTG CAC GCA GG 3'
primer 2 (reverse):		5' CCC GCT CAG AAG AAC TCG TC 3'
PCR cycles:	1X cycle	5 min at 94°C
	30X cycles	1 min at 94°C (denaturing)
		2 min at 64°C (annealing)
		3 min at 72°C (extension)
	1X cycle	7 min at 72°C

4. The final product can be stored at 4°C until analyzed, or samples of the PCR products and their *Pst*I restriction enzyme digestion products (618 bp and 173 bp) could be analyzed on a 1% agarose gel.

Western Blotting

3.2.4.1. Preparation of Protein

1. Cells grow in a monolayer. Completely aspirate supernatant and wash with PBS once.
2. Disrupt cells by lysis buffer: Add 200 μL 1X SDS/sample buffer to each 100-mm Petri dish on the top of the ice. Scrape the monolayer cells with a policeman, and collect the cell lysis to a screw-cap microfuge tube on ice.
3. Heat the protein sample at 100°C 10 for min.
4. Shear chromosome DNA with sonicator for 30 s.
5. Centrifuge 9,000*g* for 10 min at room temperature. Remove the supernatant to a fresh tube.
6. Store at –70°C.

3.2.4.2. SDS–Polyacrylamide Gel Electrophoresis of Proteins

1. Assemble the glass-plate sandwich using two clean glass plates and two 0.75-mm spacers.
2. Prepare the separating gel solution as in **Table 1**. The percentage of acrylaminde in the separating gel depends on the molecular size of the protein being separated. As a guideline, use 5% gels for SDS-denatured proteins of 60 to 200 kDa, 10% gels for SDS-denatured proteins of 16-70 kDa, and 15% gels for SDS-denatured proteins of 12 to 45 kDa.
3. Using a Pasteur pipet, apply the separating gel solution to the sandwich along an edge of one of the spacers until the height of the solution is 1 cm below the comb.

Table 1
Separating Gel

Stock Solutions (mL)	Final Acrylamide Concentration in the Separating Gel (%)				
	6	8	10	12	15
H_2O	7.9	6.9	5.9	4.9	3.4
30% acrylamide/ 0.8% bisacrylamide	3.0	4.0	5.0	6.0	7.5
1.5*M* Tris–HCl (pH 8.8)	3.8	3.8	3.8	3.8	3.8
10% SDS	0.15	0.15	0.15	0.15	0.15
10% ammonium persulfate	0.15	0.15	0.15	0.15	0.15
TEMED	0.006	0.006	0.006	0.006	0.006

Table 2
Stacking Gel

Stock Solution	Gel Volume (mL)			
	4	6	8	10
H_2O	2.7	4.1	5.5	6.8
30% acrylamide/ 0.8% bisacrylamide	0.67	1.0	1.3	1.7
1.0*M* Tris (pH 6.8)	0.5	0.75	1.0	1.25
10% SDS	0.04	0.06	0.08	0.1
10% ammonium persulfate	0.04	0.06	0.08	0.1
TEMED	0.004	0.006	0.008	0.01

4. Using another pasteur pipet, slowly cover the top of the gel with a layer(approx 1 cm thick) of H_2O.
5. Allow the gel to polymerize 30–60 min at room temperature.
6. Pour off the layer H_2O.
7. Prepare the stacking gel solution as in **Table 2**.
8. Using a Pasteur pipet, slowly allow the stacking gel solution to trickle into the center of the sandwich along an edge of one of the spacers, until the height of the solution reach the top of the plates.
9. Insert a 0.75-mm comb into the layer of the stacking gel solution.
10. Allow the stacking gel solution to plymerize 30–45 min at room temperature.
11. Heat the samples to be separated 5 min at 100°C. Mix 5 µL Rainbow™ colored protein molecular weight markers (Amersham Life Science) with 5 µL 2X SDS/ sample buffer.
12. Carefully remove the Teflon comb. Rinse the wells with 1X electrophoresis buffer.
13. Fill the chamber with 1X electrophoresis buffer.

14. Load the protein samples with flat-tipped pipet. Add an equal volume of 1X sample buffer to any empty wells to prevent spreading of adjoining lanes.
15. Connect the power supply to the cell and run at 8 V/cm until the Bromphenol Blue tracking dye enters the separating gel. Then, increase to 15V/cm.
16. After Bromphenol Blue tracking dye has reached the bottom of the separating gel, disconnect the power supply.
17. Remove the glass plates from the electrophoresis apparatus and place them on a paper towel.
18. Discard the stacking gel. Carefully remove the separating gel and rinse with transfer buffer.

3.2.4.3. Protein Blotting with Semidry Systems

1. Prepare six pieces of 3MM Whatman filter paper with the same size of gel.
2. Prepare transfer membrane. Cut membrane to same size as gel plus 1–2 mm on each edge. Place into distilled water slowly, wetting the entire surface. Equilibrate 10–15 min in transfer buffer.
3. Place three sheets of filter paper saturated with transfer buffer on the anode.
4. Place equilibrated transfer membrane on the top of filter paper stack. Remove all bubbles between membrane and filter paper by rolling a test tube over the surface of the membrane.
5. Place gel on top of membrane. Gently roll a test tube over surface of gel to ensure intimate contact between gel and membrane and to remove any interfering bubbles.
6. Complete the transfer stack by putting the three remaining sheets of filter paper on top of the gel. Roll out bubbles as described in **step 5**.
7. Place top electrode onto transfer stack.
8. Carefully connect power supply. Transfer 1 h at 15–20 V.
9. After transfer, turn off power supply and disassemble unit. Remove membrane from transfer stack, marking orientation by cutting a corner or marking with a soft lead pencil or Paper-Mate pen.

3.2.4.4. Immunoprobing with Directly Conjugated Secondary

1. Place membrane in a plastic box with 20 mL blocking buffer. Incubate 1 h at room temperature with agitation on a shaker or 4°C overnight.
2. Dilute primary antibody in blocking buffer. For monoclonal antibodies, dilute at 1 : 2000. For polyclonal antibodies, dilute at 1 : 1000.
3. Incubate with diluted primary antibody 1 h at room temperature with constant agitation.
4. Wash three times by agitating with 200 mL TBST for 10 min each.
5. Dilute HRP–anti-Ig conjugate in blocking buffer at 1 : 2000.
6. Incubate with diluted HRP–anti-Ig for 1 h at room temperature with constant agitation.
7. Wash three times by agitating with 200 TBST for 10 min each.

3.2.4.5. Visualization with Luminescent Substrates

1. Prepare ECL Western blotting solution. Mix 1 volume ECL Western blotting reagent 1 with 1 volume ECL Western blotting reagent 2. For a 6×8-cm

membrane, 1 mL ECL Western blotting solution should be prepared.
2. Transfer membrane to ECL Western blotting solution. Soak 1 min.
3. Remove membrane. Wrap with a sheet of Saran Wrap.
4. In a darkroom, place membrane face down onto film.
5. Expose film for a few seconds to 1h.

3.2.4.6. Remove Probe from Membrane

1. Wet membrane in water for 5 min.
2. Soak in 0.2*N* NaOH for 5 min with gentle shake.
3. Wash with water for 5 min.
4. The membrane can be used for another immunological detection.

3.2.5. Analysis of RNA by Northern Blot Hybridization

Northern hybridization is used to detect the size and amount of specific mRNA molecules in preparations of total RNA. The RNA is separated according to size by electrophoresis through a denaturing agarose gel and is then transferred to a membrane support. The resulting blots are studied by hybridization analysis with labeled DNA or RNA probes. Northern blotting differs from Southern blotting largely in the initial gel fractionation step. Because they are single stranded, most RNAs are able to form secondarystructures by intermolecular base pairing and must therefore be electrophoresed under denaturing conditions. Denaturation is achieved by adding formaldehyde to the gel and loading buffers. Blotting and hybridization of RNA fractionated in an agarose–formaldehyde gel is the quickest and most reliable method for Northern analysis of specific sequences in RNA extracted from eukaryotic cells.

The ubiquity of contaminating RNases in solutions and glassware and the difficulties in ensuring that an RNA preparation remains reasonably undegraded throughout the electrophoresis, blotting, and hybridization manipulations can make it difficult to obtain good hybridization signals with RNA. To inhibit RNase activity, all solutions for Northern blotting should be prepared using sterile deionized water that has been treated with diethylpyrocarbonate (DEPC). RNA should not be electrophoresed in gel tanks previously used for DNA separations, a new tank plus accessories should be obtained and saved exclusively for RNA work.

3.2.5.1. Precautions to Avoid RNase Contamination

1. Solutions. Any water or salt solutions used in RNA preparation should be treated with DEPC. This chemical inactivates ribonucleases by covalent modification. Solutions containing Tris cannot be effectively treated with DEPC because Tris reacts with DEPC to inactivate it.
2. Glassware and plastic. Labware used in the preparation of RNA should be treated to remove residual RNase activity. Autoclaving will not fully inactivate many

RNases. Glassware can be baked at 200°C for 4 h. Plasticware can be rinsed with chloroform to inactivate RNase. Plasticware straight out of the package is generally free from contamination and can be used as is.

3. Hands are a major source of contaminating RNase. Wear gloves during the experiment.

3.2.5.2. Preparation of RNA from Eukaryotic Cells

Prepare RNA from cells using RNeasy Mini Kit (Qiagen) according to the manufacture's protocol.

3.2.5.3. Agarose–Formaldehyde Gel Electrophoresis

1. Dissolve 1.0 g agarose in 72 mL water and cool to 60°C.
2. In a fume hood, add 10 mL 10X MOPS running buffer and 18 mL 12.3*M* formaldehyde.
3. Pour the gel and allow it to set. Remove the comb, place the gel in the gel tank, and add sufficient 1X MOPS running buffer.
4. Adjust the volume of each RNA sample to 11 µL (0.5–10 µg) with water, then add the following:

   5 µL 10X MOPS running buffer
   9 µL 12.3*M* formaldehyde
   25µL formamide
   10µL loading dye

   Mix well. Incubate 15 min at 55°C. Duplicate samples should be loaded on one side of the gel for ethidium bromide staining.
5. Run the gel at 5 V/cm until the Bromphenol Blue dye has migrated one-half to two-thirds the length of the gel.
6. Remove the gel and cut off the lanes to be stained. Place this portion of gel in 0.5 µg/mL ethidium bromide in 0.5*M* ammonium acetate, and allow to stain for 1 h.
7. Examine gel on an UV transilluminator to visualize the RNA and photograph with a ruler placed alongside the gel so that band positions can later be identified on the membrane.

3.2.5.4. Transfer of RNA from Gel to Membrane

1. Place unstained portion of gel in an RNase-free glass dish and rinse with several changes of sufficient deionized water to cover the gel.
2. Add 500 mL 0.05*N* NaOH to dish and soak for 30 min. Decant and add 500 mL water with shaking for three times, 15 min each.
3. Replace solution with 500 mL 20X SSC and soak for 30 min.
4. Place a solid support with wicks made out of Whatman 3MM filter paper in a dish, fill the dish with enough 20X SSC.
5. Wet the Whatman 3MM paper with 20X SSC.
6. Place the gel on the filter paper and squeeze out air bubbles by rolling a glass pipet over the surface.
7. Cut four strips of plastic wrap and place over the edges of the gel. This is to prevent the buffer from "short-circuiting."

8. Cut a piece of Hybond^{TM} –N^{+} membrane (Amersham) just large enough to cover the exposed surface of the gel. Wet the membrane with water for 5 min.
9. Place the wetted membrane on the surface of the gel. Remove bubbles by rolling a glass pipet over the surface.
10. Flood the surface of the membrane with 20X SSC. Cut five sheets of Whatman 3MM paper to the same size as the membrane and place on top of the membrane.
11. Place stack of paper towels on top of the filter paper to a height of approx 4 cm.
12. Lay a glass plate on top of the structure and add a bottle containing 500 mL water. Leave overnight.
13. Remove paper towels and filter papers and recover the membrane and flattened gel. Mark, in pencil, the position if wells on the membrane and ensure that the up–down and back–front orientations are recognizable.
14. Rinse the membrane in 2X SSC, then place it on a sheet of Whatman 3MM paper and allow to dry.

3.2.5.5. Immobilize the RNA

1. Bake the membrane for 30 min at 80°C.
2. Wrap the dry membrane in UV-transparent plastic wrap, place RNA-side down on a UV transilluminator, Stratalinker 1800 (Stratagene).

3.2.5.6. Hybridization Analysis

1. Prepare DNA probe with HYPER-PRIMER Quick kit (Bioline) according to manufacture's protocol. Purify the probe through MicroSpinTM G-50 column (Pharmacia Biotech).
2. Wet the membrane carrying the immobilized RNA in 5X SSC.
3. Place the membrane RNA-side up in a hybridization tube, add 10 mL Rapid-Hyb buffer (Amersham).
4. Place the tube in the hybridization oven and incubate with rotation 1 hour at 65°C.
5. Heat the probe for 10 min at 100°C. Cool in ice for 1 min.
6. Pipet the probe into the hybridization tube and continue to incubate with rotation at 65°C for 1 h.
7. Pour off hybridization solution and add an equal volume of 2X SSC/0.1% SDS. Incubate with rotation 10 min at 65°C.
8. Replace wash solution with an equal volume of 0.2X SSC/0.1% SDS and incubate 10 min at 65°C with rotation.
9. If desired, carry out two further washings using 0.2X SSC/0.1% SDS at 65°C for 10 min with rotation.
10. Remove final wash solution and rinse membrane in 2X SSC at room temperature. Wrap with UV-transparent plastic wrap.
11. Perform autoradiography.

3.2.5.7. Remove Probe from Membrane

1. Boil the membrane with 500 mL stripping buffer, 5min at 100°C.
2. If desired, shake the membrane with hot stripping solution for further 30 min.

3. Rinse the membrane with 2X SSC.
4. The membrane can be used for another probe hybridization analysis.

3.3 Clinical Application

An investigational study involving nine patients with HCC was performed using intratumoral injection of pDNA encoding p53 ***(9)***. Inclusion criteria were histological proof of malignancy, normal coagulation profile, and the presence of unresectable tumor. Patients were informed of and had to consent to the experimental nature and unproven results of the procedure. Patients were hospitalized for the injection and for 24 h following, during which they were monitored for pulse, blood pressure, urine output, and central venous pressure. The patients had percutaneous intratumoral injection under ultrasound or computed tomography (CT) scan control. In the first four patients a single injection of 2 mg of ''naked'' DNA was performed and the remaining five patients had four daily injections of 0.5 mg DNA complexed with liposomes.

The CT scan and serum α-fetoprotein (AFP) were measured at 4 wk after the injections and compared to pretreatment measurements. A positive response was defined as a decrease in the level of AFP or tumor size on the CT scan.

There was no mortality or morbidity as a result of the injections. Some patients who received naked DNA injections showed transient fever, hypertension, and hypotension. Four patients showed objective tumor response.

Because of the short follow-up and the small number of patients, it is difficult to comment on the effectiveness of this approach. The mechanism of growth inhibition in the four patients remains unclear because there was no analysis of gene transfer and expression, as postinjection biopsies were not performed.

There is an ongoing Phase I trial using regional infusion of adenoviral p53 that has shown no significant adverse effects even at quite a high dosage (personal communication, Dr. Alan Venook University of California, San Francisco). We have approval for treatment of liver cancer with regional adenoviral p53 infusion and expect to enroll the first patients in the near future.

4. Notes

1. All the tips, tubes, glassware, and disposables should be sterile.
2. The greatest care must be taken not to cause contamination. Carry out work close to a Bunsen burner to kill bacteria in the air, when necessary.
3. The antibiotic added to culture media depends on the antibiotic resistance genes encoded by the plasmid.
4. Plasmids are usually provided in small aliquots dissolved in TE buffer.
5. Metal inoculation loops should be burnt to red by the Bunsen burner in order to prevent contamination and should be cooled down by touching the surface of the LB–agar (avoiding colonies).
6. Do not forget to label cryo-tubes before preparation of glycerol stock.

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Antisense IGF-I for Hepatocellular Carcinoma

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1. Introduction

Antisense gene therapy involves the selective inhibition of expression or function of disease-causing genes by treatment with an antisense molecules (RNA or oligodeoxynucleotides) complementary to its target nucleic acids, usually to some specific mRNA molecules or mRNA precursor molecules, at a specific site ***(1,2)*** *(see also* **Note 1**).

To be effective, it is necessary to get an adequate concentration of antisense oligodeoxynucleotides in the same subcellular compartment as the target RNA without damaging the cell membrane. While on active transcription, every gene is transcribed into 10^2–10^4 copies of mRNA, which, in turn, are translated into 10^4–10^6 copies of protein molecules. It makes the synthesized antisense oligodeoxynucleotides too expensive as therapeutic drugs. A promising alternative may be the antisense RNA ***(1,3)***.

Antisense RNA containing the complementary sequence to a given mRNA are transcripts of antisense genes. It has the potential for continuous production of antisense molecules with simple design. Commonly, cells are transfected by a vector that contains the sequence that transcripts the effective RNA. This approach provides a permanent supply of the therapeutic RNA after the vector has been successfully implemented. The formation of sense–antisense mRNA–RNA hybrids could, in principle, prevent transcription of the authentic gene, processing of its RNA product, or translation of the messenger; the RNA–RNA duplexes may serve as substrates for RNaseIII and a deaminases, thus directly destabilizing the target mRNA; it may also activate enzymes of the interferon-associated antiviral pathway ***(4–6)***.

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

Most antisense RNA strategy is ex vivo approach. A vector containing the antisense RNA sequence is designed and constructed. The vector is then transfected in vitro into the target cells, which are isolated from the individual considered for gene therapy (e.g., tumor cells, tumor-infiltrating lymphocytes, hematopoietic stem cells, hepatocytes, and others). After in vitro selection and characterization, the genetically modified autologous cells are returned to the patient.

The great majority of primary liver cancers are hepatocellular carcinomas (HCCs), which are multistep diseases where many genomic changes occur as a result of uncontrolled proliferation of hepatocytes. There are increasing efforts to use the antisense techniques to understand and to attack the growing number of known specific genes involved in HCC. The antisense strategy can also facilitate more precise predictions of prognosis and responses to specific forms of therapy. The role of candidate genes in the pathogenesis of HCC can be studied by inhibition of the specific overexpressed target gene with antisense RNAs.

Insulinlike growth factor l (IGF-I) is associated with the normal differentiation of mesenchymal tissues and the growth of animal and human tumors. Many animal and human malignancies, including hepatocellular cancer, have been reported to express high levels of IGF-I. In this chapter, we outline the preparations required to carry out laboratory experiment of IGF-I antisense gene therapy for hepatocellular cancer. It includes the design and construction of antisense RNA vector, the gene-transfer method using liposomes, the analysis of the inhibition of the expression of the IGF-I gene, and the effect of the approach in vivo.

2. Materials

2.1. Construction of the Episome-Based Plasmid pAntiIGF-I

1. A diagrammatic representation of the steps employed to assemble the vector is shown in **Fig.1**. The pMK' plasmid containing the mouse metallothionein I promoter fused to herpes simplex thymidine kinase was kindly provided by Richard Palmiter ***(5)***. pIGF-I harboring a human hepatic cDNA for IGF-I was kindly provided by Martin Jansen ***(5)***. pUC18 was kindly provided by Peter Harte ***(4)***.REP-4 plasmid contributes two genes from the EBV to pAntiIGF-I, the origin of replication (EBV ori-P) and the EBV-encoded nuclear antigen I, which drives the EBV ori-P's replicative function. These two genes allow pAntiIGF-I to replicate as an episome. pBluescript KS^+ (Stratagene) plasmid was obtained commercially.
2. DH5α host bacteria.
3. *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I with buffers.
4. T4 DNA ligase with buffer.
5. Klenow fragment with buffer
6. QIAquick Gel Extract kit (QIAgene).
7. Centrifuge.
8. CO_2 Incubator.

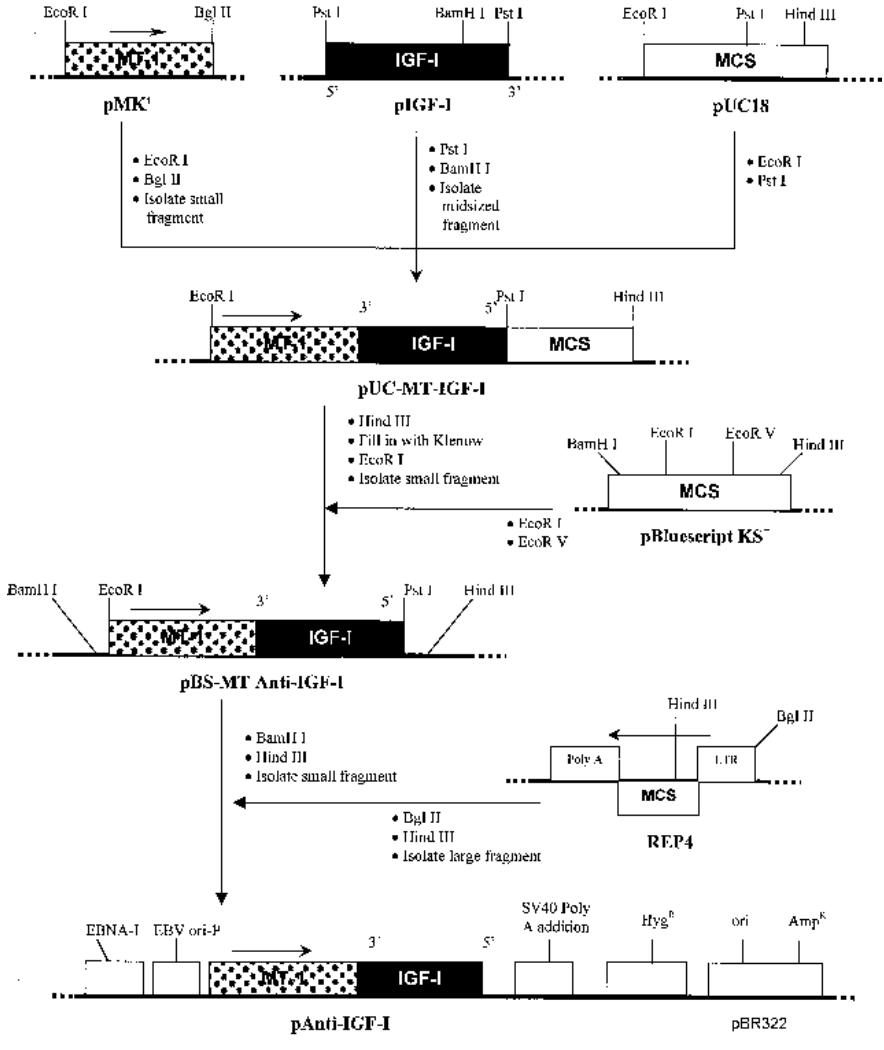

Fig. 1. Diagrammatic representation of the steps employed to construct the episomal vector pAnti-IGF-I. MT-I, metallothionein-I; MCS, multiple cloning site; LTR, long terminal repeat; EBNA-I, EBV-encoded nuclear antigen I; SV40, simian virus 40; AmpR, ampicillin resistance gene; HygR, hygromycin resistance gene.

2.2. Transfection of Tumor Cells by pAntiIGF-I and Selection of Stable Transfectants In Vitro

1. The hepatocyte or human HCC cell lines (e.g., HepG2, 7721, GW1-7, etc.).
2. Six-well and 12-well cell-culture plates.
3. RPMI-1640 cell-culture medium, store horizontally at 4°C until it is to be used.

4. Fetal bovine serum (FBS), store horizontally at –20°C until it is to be used.
5. Sterile water, filter deionized distilled water through a 0.2-µm filter, store at room temperature.
6. Appropriate antibiotics such as ampicillin, amphotericin B, and strptomycin, dissolved together in sterile water, filter it through a 0.2-µm filter and store at 4°C.
7. 0.25% trypsin aqueous solution, pH 7.2 (regulated with $NaHCO_3$); store at 4 °C.
8. 5.6% $NaHCO_3$, dissolved $NaHCO_3$ powder in sterile water, filter it through a 0.2-µm filter and store at 4°C.
9. Phosphate-buffered saline (PBS), filter it through a 0.2-µm filter and store at 4°C.
10. LIPOFECTAMINE™ Reagent (Gibco-BRL). This reagent is a 3 : 1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-l-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water. Cap ends and store horizontally at 4°C.
11. Hygramycin, dissolved in sterile water (100 µg/µL), store at 4°C.

2.3. Analyzing the Inhibitory Effect

2.3.1. Northern Blot Analysis of the Transfected Cells

2.3.1.1. Isolation of RNA by TRIZOL Extraction

1. TRIZOL reagent (Gibco-BRL). This reagent is a monophasic solution of phenol and guanidine isothiocyanate, store at 4°C.
2. Chloroform (without any additives, such as isoamyl alcohol).
3. Isopropyl alcohol.
4. RNase-free autoclaved water (diethylpyrocarbonate [DEPC] treated).
5. 75% ethanol (in DEPC-treated water).
6. RNase-free glass bottles and Eppendorf tubes.
7. Centrifuge.

2.3.1.2. Labeling the Probes Using *redi*prime DNA Labeling System

1. *redi*prime DNA labeling system (Amersham Inc.).
2. [^{32}-P]dCTP, 3000 Ci/mmol, obtained commercially, store at 0°C.
3. Incubator (37°C) and heater.
4. Linear DNA probe.
5. Sterile water.
6. Centrifuge and Eppendorf tubes.

2.3.1.3. Detection of Inhibition of the Target mRNA by Northern Hybridization

1. Diethylpyrocarbonate (DEPC).
2. 10X and 1X MOPS running buffer. 10X: 0.4*M* MOPS [3-(*N*-morpholino)-propanesulfonic acid], pH 7.0, 0.1*M* sodium acetate, 0.01*M* EDTA.
3. 12.3*M* (37%) formaldehyde, pH>4.0.
4. RNA sample: total cellular RNA **(Subheading 3.3.1.1.)** and DNA probes **(Subheading 3.3.1.2.)**.

5. RNA markers.
6. Formamide.
7 6X formaldehyde loading buffer: 1 m*M* EDTA, pH 8.0; 0.25% (w/v) Bromphenol Blue; 0.25% (w/v) xylene cyanal; 50% (v/v) glycerol.
8. 0.5*M* ammonium acetate and 0.5 µg/mL ethidium bromide in 0.5*M* ammonium acetate or 10 m*M* sodium phosphate (pH 7.0)/1.1*M* formaldehyde with and without 10 µg/mL Acridine Orange.
9. 20X, 2X, and 6X SSC.
10. Prehybridization/hybridization solution: 7% sodium dodecyl sulfate (SDS), 0.5*M* NaH_2PO_4, 0.5*M* Na_2HPO_4, 10 mM EDTA, 1% bovine serum albumin (BSA).
11. Wash solution A: 0.5% BSA, 5% SDS, 1 m*M* EDTA, 40 m*M* Na_2HPO_4, 40 m*M* NaH_2PO_4; Wash solution B: 1% SDS, 1 m*M* EDTA, 40 m*M* Na_2HPO_4, 40 m*M* NaH_2 PO_4.
12. 60°C water bath.
13. Whatman 3MM filter paper sheets.
14. Nitrocellulose or nylon membrane.
15. UV transilluminator, calibrated.
16. Hybridization oven (e.g., Hybridiser HB-1, Techne).

2.3.2. Western Blot Analysis of the Transfected Cells

2.3.2.1. Isolation of Protein from the Target Cells

1. Isopropyl alcohol.
2. 0.3*M* guandine hydrochloride in 95% ethanol.
3. Ethanol.
4. 1% SDS.
5. Centrifuge and 1.5-mL-Eppendorf tubes.

2.3.2.2. SDS–Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Protein

1. Separating gel solution (6%): 3mL 30% acrylamide/0.8% bisacrylamide, 3.8 mL 1.5*M* Tris(pH 8.8), 0.15mL 10% SDS, 0.15mL 10% ammonium persulfate, 7.9mL H_2O, 0.012 mL TEMED.
2. Stacking gel solution: 0.67 mL 30% acrylamide/0.8% bisacrylamide, 0.5 mL 1.0*M* Tris–HCL (pH 6.8), 0.04mL 10% SDS, 0.04mL 10% ammonium persulfate, 2.7mL H_2O, 0.004 mL TEMED.
3. Protein sample.
4. 2X SDS sample buffer: 100 m*M* Tris–HCl (pH 6.8), 200 m*M* dithiothreitol (DTT), 4% SDS, 0.2% Bromphenol Blue, 20% glycerol.
5. 1X Tris/glycine electrophoresis buffer: 25m*M* Tris, 250m*M* glycine, 0.1% SDS.
6. Electrophoresis apparatus with Telflon comb.
7. 25-or 100-µL syringe with flat-tipped needle.
8. Protein standards (e.g., prestained SDS-PAGE standards, Bio-Rad).

2.3.2.3. Protein Blotting with Semidry Systems

1. Transfer buffer: add 18.2 g Tris base and 86.5 g glycine to 4 L H_2O, then add 1200 mL methanol and bring to 6 L with H_2O, pH 8.3–8.4.
2. Whatman 3MM filter paper.
3. Transfer membrane (0.45 µm nitrocellulose, Amersham).
4. Semidry transfer unit (Hoefer, Bio-Rad).

2.3.2.4. Immunoprobing with Avidin–Biotin Coupling to the Secondary Antibody

1. Membrane with transferred proteins.
2. TBS: 100 m*M* Tris–HCl, pH 7.5, 0.9% (w/v) NaCl, can be store at 4°C for several months.
3. Blocking buffer (TTBS): 0.1% (v/v) Tween-20 in Tris-buffered saline (TBS), can be store at 4°C for several months.
4. Horseradish peroxidase (HRPO, Sigma)
5. Vectastain ABC (peroxidase) kit (Vector Laboratories) containing reagent A (avidin), reagent B (biotinylated HRPO), biotinylated secondary antibody.
6. Primary antibody specific for protein of interest.

2.3.3. Determination of Cell Growth

1. Cell automatic harvester.
2. Other materials are the same as those of **items 2 – 10** in **Subheading 2.2.**
3. Parental and transfected tumor cells.
4. Cells transfected with mock vector were used as a control.
5. IGF-I (R & D Systems, Minneapolis, MN).
6. [^{3}H]-thymidine (Amersham Life Science Inc., Illinois).

2.4. Measure of Tumorigenicity In Vivo

1. Nude mice and immunocompetent C57BL/6 mice.
2. Parental and transfected tumor cells.
3. 1-mL sterile syringe.

3. Methods

3.1. Construction of the Episome-Based Plasmid pAnti-IGF-I

The vector was assembled using a common molecular cloning technique **(Fig.1)**.

1. Digest the pMK' plasmid with *Eco*RI + *Bgl*II at 37°C for 5 h. Electrophorese the digestion mixture on 1% agarose gel, purify the small fragment (MT-1) from the gel using the QIAquick Gel Extraction Kit.
2. Digest the pIGF-I plasmid with *Pst*I + *Bam*HI at 37°C for 5 h. Electrophorese the digestion mixture on 1% agarose gel, purify the mid-sized fragment (IGF-I) from the gel using the QIAquick Gel Extraction Kit. *Bam*HI and *Bgl*II have different recognition sequences, but can generate identical staggered ends that are compatible for ligation to each other.

3. Digest the pUC18 plasmid with *Eco*RI + *Pst*I at 37°C for 5 h. Electrophorese the digestion mixture on 1% agarose gel, purify the linear vector DNA from the gel using the QIAquick Gel Extraction Kit.
4. Ligate these three fragments with T4 DNA ligase; incubate the mixture at 16°C for 5 h for generating pUC-MT–IGF-I.
5. Transform the DH5α host bacteria with ligation mixture, extract and check the plasmid to get adequate amount of pUC-MT–IGF-I.
6. Digest the pUC-MT–IGF-I with *Hind*III at 37°C for 5 h. Fill its end with klenow, then inactivate the klenow at 75°C for 15 min. Then, digest the DNA with *Eco*RI at 37°C for 5 h. Electrophorese the digestion mixture on 1% agarose gel; extract and purify small fragment from the gel using the QIAquick Gel Extraction Kit.
7. Digest the pBluescript KS^+ plasmid with *Eco*RI + *Eco*RV at 37°C for 5 h. Electrophorese the digestion mixture on 1% agarose gel; extract and purify the linear vector DNA from the gel using the QIAquick Gel Extraction Kit.
8. Ligate the linear vector with the small fragment obtained from **step 6** using T4 ligase; incubate the mixture at 16°C for 5 h.
9. Transform the DH5α host with ligation mixture, extract and check the plasmid to get an adequate amount of pBS-MT-Anti-IGF-I.
10. Digest the pBS-MT-Anti-IGF-I plasmid with BamH I + Hind III at 37°C for 5 hours. Electrophoreses the digestion mixture on 1% agarose gel, extract and purify small fragment from the gel using QIAquick Gel Extraction Kit.
11. Digest the REP4 plasmid with *Bgl*II + *Hind*III at 37°C for 5 h. Electrophorese the digestion mixture on 1% agarose gel, extract and purify large fragment from the gel using the QIAquick Gel Extraction Kit.
12. Ligate the large fragment with the small fragment obtained from **step 11** using T4 DNA ligase; incubate the mixture at 16°C for 5 h.
13. Transform the DH5α host with ligation mixture, extract and check the plasmid to get adequate amount of pAnti-IGF-I.

3.2. Transfection of Tumor Cells and Selection of Stable Transfectants In Vitro

For this experiment, the untransfected parental cells were used as the negative control. The vector DNA can easily be transfected into the target cells using LIPOFECTAMINE reagent.

1. In a six-well tissue culture plate, seed $(1–3)\times10^5$ cells per well in 2 mL of RPMI-1640 supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL), pH 6.8–7.4 (regulated by 5.6% $NaHCO_3$).
2. Incubate the cells at 37°C in 5% CO_2 in a CO_2 incubator until the cells are 50–80% confluent. This will usually take 18–24 h. Because transfection efficiency is sensitive to culture confluence, it is important to maintain a standard seeding protocol from experiment to experiment.
3. Prepare the following solutions in sterile Eppendorf tubes:
   Solution A: Dilute 1–2 µg of antisense vector DNA into 100 µL serum-free

and antibiotic-free RPMI-1640 medium (pH 6.8–7.4).
Solution B: Dilute 2–25 µL of LIPOFECTAMINE reagent into 100 µL serum-free and antibiotic-free RPMI-1640 medium (pH 6.8–7.4).

4. Combine the two solutions, mix gently, and incubate at room temperature for 15–45 min to allow the DNA–liposome complexes to form. The solution may appear cloudy, although this will not impede the transfection. While complexes form, rinse the cells once with 2 mL serum-free and antibiotic-free RPMI-1640 medium (pH 6.8–7.4).
5. Add 0.8 mL of serum-free and antibiotic-free RPMI-1640 medium (pH 6.8–7.4) to the tube containing the complexes. Mix gently and overlay the diluted complex solution onto rinsed cells. Avoid any antibacterial agents in the media during transfection.
6. Incubate the cells with the complexes for 12 h at 37°C in 5% CO_2 in a CO_2 incubator.
7. Following incubation, replace the medium with fresh medium containing 10% serum.
8. Passage the cells into culture medium containing 0.2 mg/mL hygromycin at 48 h following the start of transfection; maintain the selection pressure up until 10 d to obtain the stable transfected cells.

3.3. Analyzing the Inhibitory Effect (see Note 2)

A successful antisense strategy should result in decreased level of the specific mRNAs and protein in the target cells. The common methods for detecting the decrease of mRNA level and reduction of target proteins in the transfected cells are Northern blot and Western blot analyses, respectively. The two methods are most commonly used in molecular biology *(7)*.

3.3.1. Northern Blot Analysis of the Transfected Cells

3.3.1.1. Isolation of RNA by TRIZOL Extraction

RNA can be isolated quickly and with great purity using the TRIZOL reagent. This procedure is most useful and convenient for isolating RNA from cultured cells. The following protocol is from the instruction offered by Gibco-BRL, with minor modifications we employed in this experiment.

1. Lyse 10^7–10^9 cells directly by adding 1 mL/well of TRIZOL reagent to the six-well culture dish and passing the cell lysate several times through a pipet.
2. Incubate the homogenized samples for 5 min at 15–30°C to permit the complete dissociation of nucleoprotein complexes.
3. Add 0.2 mL chloroform/1 mL TRIZOL reagent. Cap sample tubes securely. Shake tubes vigorously by hand for 15 s and incubate them at 15–30°C for 2–3 min.
4. Centrifuge the samples at 12,000*g* for 15 minutes at 4°C.
5. Transfer the aqueous phase to a fresh tube, save the organic phase for isolation of protein. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 mL isopropyl alcohol/1 mL TRIZOL reagent used in **Step 1**.
6. Incubate samples at 15–30°C for 10 min and centrifuge at no more than 12,000*g* for 10 min at 4°C.
7. Remove the supernate. Wash the RNA pellet once with 75% ethanol, adding at

least 1 mL 75% ethanol/1 mL TRIZOL reagent used in **step 1**. Mix the sample by vortexing and centrifuge at no more than 7500*g* for 5 min at 4°C.
8. Air-dry the RNA pellet. Dissolve RNA in RNase-free water by passing the solution a few times through a pipet tip and incubating for 10 min at 55–60°C.

3.3.1.2. Labeling the Probes using *RediPrime* DNA Labeling System

1. Dilute the DNA to be labeled to a concentration of 2.5–25ng in 45 µL of sterile water.
2. Denature the DNA sample by heating to 95–100°C for 5 min in a boiling water bath.
3. Centrifuge briefly to bring the contents to the bottom of the tube.
4. Add the denatured DNA to the labeling mix and reconstitute the mix by gently flicking the tube until the blue color is evenly distributed.
5. Centrifuge briefly to bring the contents to the bottom of the tube.
6. Add 5 µL [^{32}P]-dCTP and mix by gently pipetting up and down four to five times.
7. Centrifuge briefly to bring the labeled mix to the bottom of the tube.
8. Incubate at 37°C for 10 min.
9. For use in hybridization, denature the labeled DNA by heating to 95–100°C for 5 min, then chill on ice.

3.3.1.3. Detection of Inhibition of the Target mRNA by Northern Hybridization

It is anticipated that the treatment of antisense IGF-I RNA can result in the complete disappearance of the endogenous IGF-I transcripts. In this experiment, parental nontransfected cells were used as the negative control. As in serum-free medium, expression of IGF-I transcripts is greatly enhanced ***(5)***. The stably transfected and nontransfected target cells were transferred to serum-free medium for 24 h before Northern hybridization analysis. As the expression of antisense vector can be induced by treatment of $ZnSO_4$, the transfected cells were divided into two groups that were incubated in serum-free medium in the absence and presence of $ZnSO_4$ (50 µM), respectively.

1. Dissolve 1.0 g agarose in 72 mL water and cool to 60°C in a water bath. When the flask has cooled to 60°C, place in a fume hood and add 10 mL 10X MOPS running buffer and 18 mL of 12.3*M* formaldehyde (*see* **Note 3**).
2. Pour the gel and allow it to set. Remove the comb, place the gel in the gel tank, and add sufficient 1X MOPS running buffer to cover the gel to a depth of approx 1 mm.
3. Adjust the volume of each RNA sample to 11 µL with water, then add 5 µL 10X MOPS running buffer, 9 µL 12.3*M* formaldehyde, and 25 µL formamide. Vortex and spin briefly in a microcentrifuge to collect the liquid. Incubate 15 min at 55°C (*see* **Note 4**).
4. Add 6X 10 µL formaldehyde loading buffer, vortex, spin to collect liquid, and load 10 µg RNA into the well. Run the gel at 5 V/cm until the Bromphenol Blue dye has migrated one-half to two-thirds the length of the gel (approx 3 h).
5. Remove the gel and cut off the "Marker Lane". Place this portion of the gel in an RNase-free glass dish, add sufficient 0.5*M* ammonium acetate to cover, and soak for 20 min. Change solution and soak for an additional 20 min to remove the

formaldehyde. Pour off solution, replace with 0.5 µg/mL ethidium bromide in 0.5*M* ammonium acetate, and allow to stain for 40 min.

6. Examine gel on an UV transilluminator to visualize the RNA and photograph with a ruler placed alongside the gel so that band positions can be identified on the membrane later.
7. Place unstained portion of gel in an RNase-free glass dish and rinse with several changes of sufficient deionized water to cover the gel to remove the formaldehyde.
8. Place an oblong sponge slightly larger than the gel in a glass or plastic dish. Fill the dish with enough 20X SSC to leave the soaked sponge about half-submerged in buffer.
9. Construct the transfer stack. Cut three pieces of Whatman 3MM paper to the same size as the sponge. Place them on the sponge and wet them with 20X SSC. Place the gel on the filter paper and squeeze out air bubbles by rolling a glass pipet over the surface. Cut four strips of plastic wrap and place over the edges of the gel.
10. Cut a piece of nitrocellulose membrane just large enough to cover the exposed surface of the gel. Wet the membrane with distilled water. Replace the water with 20X SSC and leave for 10 min.
11. Place the wetted membrane on the surface of the gel. Remove air bubbles. Flood the surface of the membrane with 20X SSC. Cut five sheets of Whatman 3MM paper to the same size as the membrane and place on top of the membrane. Cut paper towels to the same size as the membrane and stack on top of the Whatman 3MM paper to a height of approx 4cm.
12. Lay a glass plate on top of the stack and add a weight (0.5 kg) to hold everything in place. Leave overnight.
13. Disassemble transfer stack, recover the membrane, and flattened the gel. Mark the position of the wells on the membrane in pencil and mark the orientation of the gel.
14. Rinse the membrane in 2X SSC, then, place it on a sheet of Whatman 3MM paper and allow to dry completely.
15. Place the membrane between two sheets of Whatman 3MM filter paper and bake under vacuum 0.5–2 h at 80°C.
16. Wet the membrane carrying the immobilized RNA in 6X SSC.
17. Place the membrane RNA-side up in a hybridization tube and add approx 1mL prehybridization/hybridization solution per 10 cm^2 of membrane. Place the tube in the hybridization oven and incubate with rotation 3 h at 68°C.
18. Pipet the ice-cold denatured probes (**Subheading 3.3.1.2.**) into the hybridization tube and continue to incubate with rotation overnight at 68°C for 16–24 h.
19. Pour off hybridization solution and wash in wash solution A, 15 min with rotation at room temperature, twice. Then wash in wash solution B, 30 min with rotation at 68°C, once.
20. Rinse membrane in 2X SSC at room temperature. Blot excess liquid and cover in plastic wrap. Autoradiograph.

21. Develop the film. Determine the relative abundance of target mRNA sequences of untransfected and transfected cells by comparing their intensities of hybridization signal.

3.3.2. Western Blot Analysis of the Transfected Cells

In this experiment, protein samples are from the same groups as that in Northern blotting hybridization.

3.3.2.1. Isolation of Protein from the Target Cells

1. Remove the remaining aqueous phase overlying the interphase **(step 5, Subheading 3.3.1.1.)** and add 0.3 mL 100% ethanol/1 mL of TRIZOL reagent used in **step 1** of **Subheading 3.3.1.1.** Mix samples by inversion. Next, store the samples at 15–30°C for 2–3 min and sediment DNA by centrifugation at 2000*g* for 5 min at 4°C.
2. Precipitate proteins from the phenol–ethanol supernate with isopropyl alcohol. Add 1.5 mL isopropanol/1 mL TRIZOL reagent used in **step 1** of **Subheading 3.3.1.1.** Store samples for 10 min at 15–30°C and precipitate the protein at 12,000*g* for 10 min at 2–8°C.
3. Remove the supernate and wash the protein three times in a solution containing 0.3*M* guanidine hydrochloride in 95% ethanol. Add 2 mL wash solution/1 mL TRIZOL reagent used in **step 1** of **Subheading** 3**.3.1.1.** During each wash cycle, store the protein pellet in the wash solution for 20 min at 15–30°C and centrifuge at 7500*g* for 5 min at 2–8°C. After the final wash, vortex the protein pellet in 2 mL of ethanal. Store the protein pellet in ethanol for 20 min at 15–30°C and centrifuge at 7500*g* for 5 min at 2–8°C.
4. Vacuum dry the protein pellet for 5–10 min. Dissolve it in 1% SDS at 50°C by pipetting. Sediment any insoluble material by centrifugation at 10,000*g* for 10 min at 2–8°C and transfer the supernate to a fresh tube. The sample is ready for use in Western blotting.

3.3.2.2. SDS-PAGE of Protein

1. Assemble the glass–plate sandwich of the electrophoresis apparatus according to the manufacturer's instructions.
2. Prepare the separating gel solution and degas. After adding the 10% ammonium persulfate and TEMED to the degassed solution, stir gently to mix.
3. Immediately apply the separating gel solution to the sandwich until the height of the solution between the glass plates is approx 11 cm.
4. Slowly cover the top of the gel with a layer (approx 1 cm thick) of 0.1% SDS. Allow the gel to polymerize 30–60 min at room temperature.
5. Pour off the layer of 0.1% SDS and rinse with deionized water.
6. Prepare the stacking gel solution. Slowly allow the stacking gel solution to trickle into the center of the sandwich until the height of the solution in the sandwich is approx 1 cm from the top of the plates.
7. Insert a clean Teflon comb into the layer stacking gel solution. Allow the stacking gel solution to polymerize 30–45 min at room temperature.

8. Dilute a portion of the protein sample 1 : 1 (v/v) with 2X SDS sample buffer and heat 3–5 min at 100°C in a sealed screw-cap eppendorf tube. Dissolve protein standards mixture in 1X SDS sample buffer according to the supplier's instructions as a control.
9. Carefully remove the Teflon comb. Rinse wells with deionized water and fill the wells with 1X Tris–glycine electrophoresis buffer.
10. Load the protein samples at the same concentration into the wells using a 25-or 100- μL syringe with a flat-tipped needle.
11. Connect the power supply to the electrophoresis apparatus. Run at 8 V/cm until the Bromophenol Blue tracking dye enters the separating gel. Then, increase the voltage to 15 V/cm and run until the Bromophenol Blue tracking dye has reached the bottom of the separating gel.
12. Turn off the power supply and disconnect the gel.
13. Orient the gel so that the order of the sample wells is known. Cut off the unwanted portion of the gel according to the prestained protein standards.

3.3.2.3. Protein Blotting with Semidry Systems

1. Prepare transfer membrane. Cut membrane to same size as gel plus 1–2 mm on each edge. Wet the nitrcellulose membrane with distilled water. Equilibrate 10–15 min in transfer buffer.
2. Assemble the transfer stack: three sheets of filter paper saturated with transfer buffer, equilibrated transfer membrane, gel, and three sheets of filter paper. Roll out bubbles as each component is added to the stack.
3. Place top electrode onto transfer stack. Connect to the power supply (0.8mA/cm^2 of gel area). Transfer for 1 h, keeping the temperature lower than 45°C.
4. Turn off power and disassemble unit. Remove membrane from transfer stack, marking orientation. Proceed with immunoprobing.

3.3.2.4. Immunoprobing with Avidin–Biotin coupling to the Secondary Antibody

1. Equilibrate membrane in TTBS with constant agitation using an orbital shaker or rocking platform. Incubate 30–60 min at room temperature.
2. Prepare primary antibody solution in TTBS.
3. Remove membrane from TTBS and place in enough primary antibody solution to cover membrane. Incubate 30 min at room temperature with gentle rocking.
4. Wash membrane three times over a 15-mm span in TTBS.
5. Prepare biotinylated secondary antibody solution by diluting two drops biotinylated antibody with 50–100 mL TTBS.
6. Transfer membrane to seconday antibody solution. Incubate 30 min at room temperature with slow rocking, then, wash as in **step 4**.
7. While membrane is being incubated with secondary antibody, prepare the avidin–biotin–HRPO complex. Mix 2 drops Vectastain reagent A and 2 drops reagent B into 10 mL TTBS. Incubate 30 min at room temperature; then, further dilute to 50 mL with TTBS.
8. Transfer membrane to avidin–biotin–enzyme solution. Incubate 30 min at room

temperature with slow rocking; then, wash over a span as in **step 4**.
9. Wash membrane 15 min at room temperature in 50 ml TBS to remove excess phosphate and Tween-20.
10. Place membrane into HRPO-based chromogenic visualization solution. Bands should appear in 10–30 min.
11. Terminate reaction by washing membrane in distilled water. Air-dry and photograph for a permanent record. Determine the relative abundance of target protein of transfected and nontransfected cells by comparing their intensities of immunoblotting signal.

3.3.3. Determination of Cell Growth

It is expected that the antisense IGF-I RNAs can inhibit the growth of tumor cells by correcting the overexpressed target gene that involved in the growth. Moreover, if the inhibition appears, it should be able to be overcome by the addition of IGF-I.

1. IGF-I-containing medium prepared by the addition of 15% conditioned medium from parental-cell-culture supernatant was used as a control.
2. Seed 1×10^4 of the parental cells, cells transfected with mock vectors, and antisense cell lines in triplicate wells in six-well plates in complete RPMI-1640 medium with or without IGF-I ***(8)***.
3. Incubate the cells at 37°C, in 5%CO_2 in a CO_2 incubator for 24 h.
4. Pulse cells with 1μCi of [^{3}H]-thymidine for 4 and 12 h.
5. Collect cells on a glass–fiber filter with cell harvester.
6. Rinse the cells with PBS and fix with methanol.
7. Determine the [^{3}H]-thymidine incorporation using a radioactive counter. Count out the cell proliferation curve.

3.4. Measure of Tumorigenicity In Vivo

The tumorigenic capacities of control and antisense mRNA transfected cell lines can be assayed by subcutaneous injection of cells into immunodeficient nude mice and immunocompetent C57BL/6 mice. It is desired that the antisense IGF-I can also have some therapeutic effect on the established tumors by elicit specific immune reaction in immunocompetent C57BL/6 mice ***(6,9)***.

1. Breed the nude mice and C57BL/6 mice to 4 wk old. Five mice for each group.
2. Subcutaneous injection of 1×10^6 cells (parental or transfected) into two sites in opposite flanks of nude mice and C57BL/6 mice. Wear sterile gloves during the manipulation.
3. Routinely observe the animals for visible tumor growth.
4. Injection of 1×10^6 transfected cells into the formed tumor in the mice.
5. Observe the animals for visible tumor growth and evaluate the therapeutic effect of antisense IGF-I.

4. Notes

1. Besides liposome-mediated transfection, there are several methods of delivering antisense gene therapy for HCC currently:

   Naked DNA: Both antisense RNAs and antisense oligodeoxynucleotides (AS–ODNs) can be administered directly into tissue by injection and may also be incorporated into cultures cells through endocytosis by coincubation. Although the manipulation is relatively easy, the disadvantages of this procedure are as follows: difficult to control sites of uptake, some tissues are preferential (e.g., liver, kidney, blood vessels), and DNA copy number is unquantifiable. So far, many researches have used this method successfully.

   Liposome-encapsulated oligonucleotides: In this technique, unquantifiable DNA copy number, preferential uptake in certain tissues (e.g., liver), and toxicity in some tissues (e.g., brain) are the major disadvantages. Of course, it is easy to produce and also injectable, it may also facilitate uptake and provide longer-lasting stability. Liposome development with cationic lipids also allows high transfection efficiency of plasmid DNA and short, single-stranded AS–ODNs. Thus, it not only simplifies the production of antisense delivery system, but also allows for a variety of routes of delivery, including aerosol nasal sprays and parenteral injections. It is the promising pathway in antisense technique.

   Viral vectors: Currently, there are several viruses that have been used for gene delivery in antisense gene therapy (i.e., retrovirus, adenovirus, adeno-associated virus and herpes simplex virus) and they are especially useful for expression of antisense sequences with recombinant vectors. Unfortunately, although each has its advantages, none can fulfill the criteria of the "ideal viral vector." From a practical point of view, the ideal viral vector for antisense gene therapy should be sufficiently stable and safe in serum and would deliver a defined gene copy number into each infected cell. In addition, it must be able to penetrate into the cell efficiently, especially the target cell, and inside the cell to carry out the desired inhibitory effect. To put the vector into practice, it should be easy to manipulate and produce in pure form. A number of protocols are now available.
2. There are many methods can be used to detect the inhibitory effects, depending on the type and the specific function of the target gene.
3. Formaldehyde is toxic through skin contact and inhalation of vapors. All operations involving formaldehyde should be carried out in a fume hood.
4. Formamide is a teratogen and should be handled with care.

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Novel Effective Tumor Vaccines for Hepatocellular Carcinoma

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies in numerous countries. The incidence of HCC is rapidly increasing and proximately results in 350,000 deaths each year worldwide ***(1–4)***. The conventional chemotherapy and radiation treatment for HCC have been disappointing, with an overall 5-yr survival rate of less than 5%. Although the surgical excision is considered the most effective therapy for early-stage HCC patients, only a very small proportion of patients with an operable primary lesion may transiently benefit from surgical treatment because of a high recurrence rate of cancer after operation. Other therapeutic approaches, such as local alcohol injection, hepatic arterial immobilization, and other alternative therapies, have little impact on this malignancy. These results highlight the urgent need for new therapies for HCC treatment.

Recently, immunotherapy has become a critically important component of clinical cancer therapies and has the potential to prevent tumor recurrence and to prolong survival of patients receiving conventional treatment for malignant disease. The major concept of these approaches is to artificially enhance the immunogenicity of tumor cells ***(5,6)***.

Many tumor cells process antigens that the host system can recognize and elicit an immune response in classical transplantation tests. T-cells rather than B-cells play an important role in the host antitumor responses. Central to the activation of T-cells is the interaction between the clonally restricted T-cell receptor and the peptide– major histocompatibility complex (MHC) displayed

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

on antigen-presenting cells (APCs). The initial encounter between a T-cell and APC also involves adhesion molecules and requires costimulatory signals that are provided by the APCs. One of the best characterized costimulatory molecules is CD28 presented on T-cells. The ligands for CD28 are B7-1 and B7-2, which are presented on activated APCs. Crosslinking CD28 molecules on T-cells by anti-CD28 Mab can deliver a costimulatory signal both in vitro and in vivo ***(7–10)***.

Tumor rejection is primarily mediated by cytotoxic T-cells. For activation of $CD8^+$ CTLs, tumor antigens must be intracellularly processed into small peptides and presented through MHC class I molecules on the cell surface. Escape from immune surveillance may occur because the tumor cells may lack some components that are essential for the activation process. For example, tumor cells may modulate tumor antigens to avoid immune surveillance. A downregulation or loss of MHC molecules has also been implicated as a mechanism for tumor escaping from host immunity. In addition to the antigen-specific signal delivered by interaction of the MHC–peptide complex with the T-cell receptor, the second costimulatory signal, mediated by the B7/CD28 counterreceptor pathway has a key role in T-cell activation. The confirmation that B7 molecule is important for tumor rejection has been directly demonstrated by gene transfection experiments. Transfection of the B7-1 or B7-2 genes into low immunogenic tumor cell lines can increase immunogenicity ***(11)***.

Several approaches have been used to generate immunogenic tumor cells. Recent advances in genetic engineering allow for the modification of tumor cells by gene transfection. It has been shown that transfection of MHC genes into tumor cells can convert nonimmunogenic tumor cell lines into immunogenic ones. Immunizations of animals with tumor cells transfected with various cytokine genes also enhance host antitumor immune responses. Several recent studies have demonstrated that in the absence of an exogenous help, alloreactive CTLs can be generated in vitro by stimulation of naive $CD8^+$ T-cells with B7-1 transfected tumor cells. However, stimulation of naive T-cells with B7 transfected tumor cells may be problematic. It has been speculated that spontaneously derived tumor cells may lack tumor antigen–MHC complexes on their cell surfaces. Moreover, primary tumor cells are very difficult to transfect. In addition to costimulatory molecules, adhesion molecules are also important in the activation of T-cells. Treatment of tumor cells in vitro with interferon-γ(IFN-γ) and tumor necrosis factor-α(TNF-α) can significantly increase the expression of ICAM-1 and other adhesion molecules.

An effective tumor vaccine should have all the signals essential for T-cell activation. Activated B-cells are the most effective APCs. In addition to expressing high levels of MHC class I and II antigens, activated B-cells also express high levels of accessory and costimulatory molecules. We fused BERH-2 cells, a chemically induced rat hepatoma cell line, with in vivo

activated B-cells. The hybrid tumor cells expressed high levels of MHC class I and class II, ICAM-1, and B7 and lost tumorigenicity in syngeneic animals. Rats immunized with the hybrid cells became resistant to parental tumor cell challenge and the hybrid cells were also confirmed to be able to cure established tumors ***(12)***. Several recent articles have shown that autologous dendritic cells pulsed in vitro with tumor antigens including tumor-associated-antigen were able to be used as tumor cellular vaccines to stimulate host immunity ***(13,14)***. In contrast to gene transfection experiments in which only one or two genes can be introduced into tumor cells at a time, fusion of tumor cells with APCs or dendritic cells pulsed with tumor antigens gives rise to modified APCs with both tumor-specific and costimulatory signals ***(15)***. However, these approaches are still time-consuming and problematic because of requiring large number of activated B-cells and dendritic cells. In view of these results and problems, our research goal has been to develop a strategy with simplicity and broad clinical applicability for the generation of cellular cancer vaccines ***(1,6,17)***.

We have developed a two-step process for generating cellular tumor vaccine ***(6)***. The first step is to amplify antigen-specific and costimulatory signals by the treatment of tumor cells in vitro with a combination of cytokines. This results in enhanced expression of MHC and adhesion molecules. The second step is to use a bi-Mab specific for both tumor antigen and CD28 molecules to provide a direct bridge to CD28 of T-cells for induction of CD28-mediated costimulation. This bridge also facilitates interaction of other adhesion molecules on T-cells and tumor cells. Thus, tumor cells with both amplified antigen-specific and costimulatory signals and armed with a bi-Mab targeted to CD28 became strongly immunogenic and could be used as tumor vaccines for the induction of antitumor immunity. The enhanced immunity in two animal model systems protected against parental tumor cell challenges and was able to cure established tumors. The two-step approach for development of effective tumor vaccines has the potential to become applicable to a broad spectrum of cancers. This is because the step in the procedure that bridges antigen on the tumor cells to a key costimulatory molecule, the CD28 of T-cells, is done in vitro and can be locked into place. Thus, antigens on tumor cells need not be unique. Therefore, there may be no need to define tumor-specific transplantation antigen (TSTA). To advance this approach to clinical human trials, which is ultimately significant to cancer immunotherapy, we will need to know mechanism(s) in order to optimize the treatment. We will also need to know if this approach is effective in different human cancer model systems and if it can be made useful for treatment of human cancers clinically. Importantly, we will further investigate if effective cancer vaccines can be generated in vivo by direct intratumor injection of cytokines and bi-Mabs.

2. Materials

2.1. Rat Hepatoma Cellular Vaccine Generated by Fusion of Tumor Cells with Activated syngeneic B-Cells

1. Rat: Wistar rat, male, 6–8 wk of age. (*see* **Note** 2).
2. BERH-2 cell: a chemical carcinogen-induced hepatocarcinoma from the Wistar rat ***(6)***. Cells derived from this tumor grow rapidly and form tumors in the liver of syngeneic animals. The cells have been maintained in regular culture conditions for more than 5 yr.
3. Freund's complete adjuvant.
4. RPMI-1640 medium: prewarmed to 37°C before use (Gibco-BRL).
5. Dulbecco(s phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} at pH 7.4 (Gibco-BRL).
6. Collagenase type IV, DNAse type I (Sigma, St. Louis, MO).
7. Polyethylene glycol (PEG) 1400 MW (Boehringer Mannheim, Indianapolis IN).
8. Flow cytometery: FACScan® (Becton Dickinson, San Jose, CA).
9. Monoclonal antibodies: mouse antibodies to rat MHC class I (OX-18) and class II (OX-6), ICAM-1 (IA29), or LFA-1 (WT.1), were prepared by us (data not published).
10. CTLA4-Ig: a soluble fusion protein containing the variable domain of the human CTLA-4 protein and the hinge, CH2, and CH3 domains of the human IgG1 constant region. Our CTLA4-Ig was a generous gift from Professor Lieping Chen ***(9)***.
11. Fluorescein isothiocyanate (FITC)-conjugated goat antibody to mouse Ig or FITC-labeled rabbit antibody to human Ig (ZYMED Laboratories Inc., South San Francisco, CA).
12. Negative control antibodies: mouse antibody to human CD3 (GH3, IgG2b), and a soluble human CD44-Ig chimeric protein.
13. Anti-BERH-2 tumor cell polyclonal antiserum preparation: We prepared polyclonal antiserum by injecting rabbits subcutaneously with BERH-2 hepatoma cells, mixed with Freund's complete adjuvant. After repeated boosting for 2 mo, the antisera were collected and purified by passing through a protein-G column. Finally, the antisera were repeatedly absorbed with rat B-cells (to eliminate the crossreaction of the antisera with rat B-cells extensively).
14. Anti-rat B-cell polyclonal antisera preparation: We prepared polyclonal antisera by injecting rabbits subcutaneously with purified activated B-cells from Wistar rats, mixed with Freund's complete adjuvant. Activated B-cells were purified by panning with plates coated with purified goat antibody to rat immunoglobulin (IgG). After repeated boosting for 2 mo, the antisera were collected and purified by passage over a protein-G column. Finally, the antisera were repeatedly absorbed with BERH-2 hepatoma cells (to eliminate the crossreaction of the antisera with tumor cells).

2.2. Mouse Hepatoma Vaccine Generated by Fusion of Tumor Cells with Syngeneic Dendritic Cells

1. Mouse: C57BL/6 mouse, male, 4–6 wk of age.

2. Hepa 1–6 tumor cell: from a chemically induced hepatoma from the C57BL/6 mouse ***(18)***. (*see* **Note 3.**)
3. Medium: DMEM medium (Gibco-BRL) supplemented with 10% fetal calf serum (FCS).
4. Cytokines: recombinant mouse IL-4 (Pharmingen), recombinant mouse GM-CSF (R&D Systems), both reconstituted to 10 μg/mL with sterile phosphate-buffered saline (PBS) and stored under –20°C in aliquot.
5. Anti-mouse mAbs: anti-MHC class II, anti-Mac3, anti-CD8α, anti-CD45R/B220, anti-CD3ε, anti-Gr-1.
6. Guinea pig complement: fresh-frozen pooled guinea pig serum (Gibco-BRL).

2.3. Mouse Hepatoma Cellular Vaccines Generated by In Vitro Modification of Tumor Cells with Cytokines And BsAbs (Two-Step Tumor Vaccine)

1. Mouse: C57BL/6 mouse, male, 4–6 wk of age.
2. Hepa 1–6 tumor cell: *see* **Note 3**.
3. RPMI-1640 medium, supplemented with 10% FCS, 2 m*M* glutamine, 1+ nonessential amino acid, 1 m*M* sodium pyruvate, and 20 μg/mL gentamycin. Prewarm the medium to 37°C before use.
4. Cytokines: recombinant mouse Interleukin-2 (IL-2), IFN-γ and TNF-α (R & D Systems, Minneapolis, MN), reconstituted to 10 μg/mL with sterile PBS and stored under –20°C in aliquot.
5. Anti-CD28 : anti-Hepa 1–6 bispecific monoclonal antibodies (BsAbs): CD28 : gp55, CD28 : gp95, CD28 : gp210, and CD18 : gp55 BsAb as control. For their preparation, *see* **Note 4**.
6. Rat mAbs to mouse MHC-I (M1/42), MHC-II (M5/114), ICAM-1 (HA58) and ICAM-II (3C4) were prepared by us (data not published).
7. Rat mAb to mouse CD44 (KM81) (American Type Culture Collection [ATCC, Manassas, VA]); VCAM-1 (51-10C9) (PharMingen, San Diego, CA).
8. Human CTLA4-Ig fusion protein: *see* **Subheading 2.1.**
9. FITC-labeled goat antibody to rat Ig or FITC-labeled rabbit antibody to human Ig (ZYMED Laboratories Inc., South San Francisco, CA).
10. Negative-control antibodies: rat antibody to mouse CD3 (YCD3) and a soluble human CD44-Ig chimeric protein.

3. Methods

3.1 Rat Hepatoma Cellular Vaccine Generated by Fusion of Tumor Cells with Activated Syngeneic B-Cells

1. BERH-2 single-cell suspension: prepared from the tumor's immediately surgical excision. After removing fat and fibrous tissue, mince the tumors and incubate the pieces under agitation for 1–2 h at 37°C in RPMI-1640 medium containing 1 mg/mL collagenase IV and 0.02 mg/mL DNase type I. Pass the cell suspension through a tissue sieve and wash with PBS (pH 7.4) three times at room temperature. The

viability of the cells determined by dye exclusion should exceed 90%.

2. B-Cell preparation: Activated B-cells are obtained from the spleens of rats injected 14 d earlier with bovine serum albumin (BSA) in Freund's complete adjuvant.
3. Fusion of hepatoma cells with activated B-cells: (1) Warm the medium and 50% PEG solution to 37°C before fusion. (2) Mix BERH-2 cells with activated B-cells at a ratio of 1 : 10 (e.g., 10^7 : 10^8). BERH-2 cells and activated B-cells should be mixed thoroughly. (3) Wash the mixed cells once with serum-free RPMI-1640 medium at room temperature. Centrifuge the cell mixture at 400*g* for 10 min at room temperature to form a pellet. (4) After the tumor cells and activated B-cells were mixed and centrifuged, the supernatants should be aspirated completely. The pellet should be flipped loose before fusion performed. (5) The cell pellet should be agitated gently when the PEG was added. PEG should be added slowly in the process of fusion. Add 1 ml of 50% PEG 1400 MW to a 37°C water bath over a 1-min period. (6) PEG dilution and removal: Add 1 mL serum-free medium, incubate an additional minute; add 2 mL serum-free medium, incubate for 2 min; add 4 mL serum-free medium, incubate for 4 min; then, finally, add 8 mL serum-free medium or medium with up to 10% FBS. Centrifuge cells at 200*g* for 10 min and discard supernatant.
4. Selection of hybrid cells. The fused cells are enriched by selection with a rabbit antiserum to BERH-2 cells and subsequent selection with a rabbit antiserum to rat B-cells. (1) The fused cells are poured onto the dish coated with a rabbit antiserum to BERH-2 cell and incubate at 37°C for 60 min. (2) Wash the dish with PBS to remove all unbound cells. (3) Collect the bound cells, pour the cells onto the dish coated with a rabbit antiserum to rate the B-cells, and incubate at 37°C for 60 min. (4) Wash the dish with PBS to remove unbound cells and collect the bound cells (the hybrid cells).
5. Cell staining of MHC-I, MHC-II, ICAM-I and LFA-I: 10^5 cells are washed with PBS and stained with monoclonal antibodies to rat MHC class I (OX-18), MHC class II (OX-6), ICAM-1 (IA-29), or LFA-1 (WT.1). Use a mouse antibody to human CD3 (GH3, IgG2b) as negative control. Incubate with the antibodies or chimeric protein for 30 min on ice; wash cells three times in PBS. Add FITC-conjugated goat antibody to mouse Ig and incubate with the cells for another 30 min on ice. Wash and fix samples and then analyze by a FACScan. At least 5000 cells are analyzed per sample in all experiments.
6. Cell staining of B7: Human CTLA4-Ig fusion protein is used for staining the B7 molecule of the hybrid cells. Another human CD44-Ig fusion protein is used as the negative control. After incubation with the chimeric proteins for 30 min on ice, cells are washed three times in PBS. Add FITC-labeled rabbit antibody to human Ig and incubate with cells for another 30 min on ice. Samples are then washed, fixed, and analyzed by a FACScan.

3.2. Mouse Hepatoma Cellular Vaccine Generated by Fusion of Tumor Cells with Syngeneic Dendritic Cells

3.2.1. Preparation of Mouse Dendritic Cells

1. Remove two large leg bones from each leg of five mice. Clean away as much flesh as possible from each leg.

2. Using sharp scissors, clip off both tips of the bone while holding the bone with tweezers. Flush bone marrow (BM) cells from inside of bone by inserting a 25 gage 5/8 in. needle into one open end of the bone and forcing media through the opposite end of the bone by pushing the plunger of the syringe. Usually, 5 mL/bone is adequate.
3. Wash BM cells once (spin at 1200 rpm in Sorvall RT6000B in 50 mL tube). Deplete red blood cells from the culture using 9 mL sterile dH_2O followed quickly by 1 mL 10+ PBS. Add 1 volume medium and wash cells again after filtering them over a Falcon 2053 cell stainer.
4. Reconstitute pellet in 1 mL medium. Add 10 μg/mL each of purified mAbs:

Anti-MHC class II	Anti-Mac3
Anti-CD8α	Anti-CD45R/B220
Anti-CD3ε	Anti-Gr-1

   Mix and place on ice for 20 min. Wash cells once and reconstitute the pellet in 1.5 mL medium.
5. Add 0.5 mL guinea pig serum as a source of complement. Incubate in water bath (37°C) for 45 min. Wash cells once in medium after filtering them over a Falcon 2053 cell strainer.
6. Plate cells into 8–10 100-mm tissue culture dishes with 10 mL medium per plate containing 10 ng/mL GM-CSF and 1ng/mL IL-4.
7. Gently remove medium and all suspended cells (do not pipet medium over cultures to remove adherent T-cells) at d 2 of culture. Replace medium with cytokines.
8. Repeat **step 7** at d 4 of culture.
9. Harvest dendritic cells (DC) between d 5 and d 10 using gentle pipetting; approx $2–3\times10^6$ DC/mouse are expected.

3.2.2. Fusion of Tumor Cells with Enriched Dendritic Cells

1. Hepa 1–6 single cell suspension: For protocols, *see* **step 1** in **Subheading 3.1.**
2. Fuse the dendritic cells harvested at **step 2** in **Subheading 3.2**. with Hepa 1–6 cells with PEG, process similar to **Subheading 3.1.** The ratio of dendritic cells : tumor cells should be 4–8 : 1.

3.3. Mouse Hepatoma Cellular Vaccines Generated by In Vitro Modification of Tumor Cells with Cytokines and BsAbs (Two-Step Tumor Vaccine)

1. Hepa 1–6 single-cell suspension: for protocols, *see* **step 1** in **Subheading 3.1.**
2. Cytokines treatment of the tumor cells: Cells (1 mL) are plated into 24-well tissue culture plates at a concentration of 2×10^6 cells/mL in RPMI-1640 complete medium with IFN-γ (100 μ*M*/mL) and TNF-α (μ*M*/mL) added freshly. Incubate the plate in a 5% CO_2, 37°C incubator for 48 h. Incubate Hepa 1–6 cells similarly, but no cytokines are used as control.
3. Flow cytometric analysis: Cells are stained with rat mAbs to mouse MHC-I (M1/42), MHC-II (M5/114), CD44 (KM81) (ATCC), ICAM-I (HA58), ICAM-II (3C4), and VCAM-1 (51-10C9), and human CTLA4-Ig fusion protein. For

staining process, *see* **Subheading** 3.1., **steps 5** and **6**.

4. Preparation of cellular tumor vaccines in vitro: After treatment for 48 h in vitro with a combination of IFN-γ and TNF-α, cells are then washed with PBS (pH 7.4) three times at room temperature. Incubate with anti-CD28 BsAbs at a concentration of 50 μg/mL on ice for 45 min. After being washed, cells are subjected to an additional incubation in an equal volume of 30% polyethylene (PEG, *see* **Subheading 2.1.**) in RPMI-1640 at 4°C for 30 min as previously reported ***(16)***. Finally, the cells are again washed with PBS three times and suspended in a final concentration of $(1–2)\times10^7$/mL PBS.
5. Generation of CTLs and cytotoxicity assays: To stimulate tumor-specific CTL responses, purified spleen T-cells from naive C57BL/6 mice are primed with γ-irradiated (5000 rad) cytokine-treated or control antibodies for 9 d in complete RPMI-1640 medium, supplemented with 20 units/mL IL-2. The cytotoxic activity of in vitro stimulated splenocytes was determined using the ^{51}Cr release assay.

4 Notes

1. Natural cell fusion is a rare event. To generate higher fusion efficiencies, two fusion techniques, electrofusion and fusing agent-mediated fusion were developed. Nowadays, polyethylene glycol (PEG) is the agent most commonly used ***(19,20)***. In using PEG as a fusing agent, cells are thought to agglutinate, giving rise to large areas of plasma membrane contact, cell shrinkage can also be observed which is thought to increase the exposure of glycoprotein and subsequently leads to closer apposition of cell membranes. PEG can increase cell fusion and do harm to cells as well. Both the degree of cell fusion and the degree of cell damage are influenced by the concentration of the PEG solution, the molecular weight of the polymer, and the purity of the synthetic compound. The main cause for the toxicity is that the fusing concentrations are hypertonic; another reason is the toxic contaminants carried by PEG. To ensure both high fusion efficiency and hybrid cell living through the toxicity, first, although molecular weights ranging from 400 to 6000 with PEG concentration ranging from 30% to 60% (w/v) can generate cell fusion, the recommended one is 1400 MW with 50% (w/v) concentration. A successful fusion procedure should bring cells together, with an optimal frequency of interactions between the two "parent" cell types, allow fusion to occur at a sufficiently rapid rate, and cause minimal damage to the cells. The potential problems relate to the frequency of interactions between similar cells (producing useless hybrids) and cell damage. Inevitably, an agent that enables membranes to "flow" together is likely to affect membrane integrity adversely ***(21)***. Only gas chromatography purity PEG is suitable for use; finally, the time of cell exposure to PEG must not be too long ***(22)***.
2. All the animals should be treated under the guidance of *NIH Guide for the Care and Use of Laboratory Animals,* or other related rules.
3. Hepa 1–6 cell line is from a chemically induced hepatoma from the C57BL/6 mouse. Cells derived from this tumor grow rapidly, forming subcutaneous tumors in syngeneic animals. This cell line also could be cultured in vitro in 10% FCS RPMI-1640 medium.

4. Preparation of monoclonal antibodies (mAbs) and BsAbs: mAbs specific for antigens on Hepa 1–-6 tumor cells and CD28 molecules on T-cells were generated by fusion of YB2/0 with spleen cells from Wistar rats immunized with either Hepa 1–6 cells or a mouse T-cell hybridoma line that has highly expressed CD28 antigen on cell surfaces. After fusion, hybridomas producing mAbs were selected using immunofluorescent analysis for flow cytometry using the FACScan. Three mAbs separately recognized a 55-kD, a 95-kD, and a 210-kD glycoprotein expressed on most tumor cells as determined by immunoprecipitation. The anti-CD28 monoclonal antibodies that specifically stained mouse CD28 transfected cells were further characterized by immunoprecipitation technique and by in vitro T-cell proliferation and interleukin (IL-2) production assays. Hybridoma-producing anti-mouse CD18 mAb was obtained from the ATCC. BsAbs were produced using a hybrid–hybridoma technique and purified by high-performance liquid chromatography as previously reported ***(23)***.
5. We recommended the PEG 1400 MW manufactured by Boehringer Mannheim.

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Immunoglobulin Fusion Proteins as a Tool for Evaluation of T-Cell Costimulatory Molecules

Andrei I. Chapoval, Gefeng Zhu, and Lieping Chen

1. Introduction

According to the current view, an efficient T-lymphocyte activation requires not only a specific signal delivered through the engagement of a T-cell receptor (TCR) by antigenic peptide–major histocompatibility complex (MHC), but also signals provided by costimulatory molecules. Accumulating evidence indicate that one of the possible reasons for poor immunogenicity of human tumors underlies in the lack of expression of costimulatory molecules ***(1)***. Recent studies reveal that, in many cases, antigens alone, especially weak tumor antigen, are insufficient to stimulate immune responses, that is, they are ignored by immune system unless second accessory signals are provided ***(2)***.

The most well-studied costimulatory signals are provided by the interaction between the B7 family members, including B7-1 (CD80) and B7-2 (CD86) on antigen-presenting cells, with CD28 on T-lymphocytes. Transfection of tumor cells with the CD80 gene could result in rejection of CD80 negative parental tumors and in the development of long-lasting immune memory ***(3)***. Several experimental models have shown that CD80 and CD86 molecules are important for the generation of immune responses against hepatocellular carcinoma ***(4–6)***.

During the last 10 yr, several dozens of new costimulatory molecules, including 4-1BB (CD137), CD40, OX40, CD27, and ICOS, have been identified. With a predicted completion of human genome sequencing by the end of 2003 ***(7)***, many new molecules with potential costimulatory properties will be available. The question that researchers and physicians will need to answer is, "What molecules have the maximum costimulatory activity and, therefore, are the most suitable for therapeutic applications?"

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

There are three major approaches for evaluation of costimulatory properties of new molecules. All of them are based on ligation of costimulatory receptors (e.g., CD28) either by natural ligand(s) (e.g., CD80, CD86) or specific monoclonal antibodies (mAb). The first approach utilizes the transfection of a full-length gene of costimulatory ligand to the immortal tumor cell line. The ability of transfected cells to augment T-lymphocyte activation after T-cell receptor (TCR) engagement is then evaluated. The second approach employs culturing of T-cells in the presence of Ag stimuli with mAb specific to the costimulatory receptor. Although this method is attractive, the costimulatory receptor is often not known and the mAb against the receptor are not available. In this chapter, we describe another strategy for the evaluation the functional activities of costimulatory molecules using soluble fusion protein between the extracellular portion of costimulatory ligand and the Fc portion of mouse IgG2a.

Fusion proteins are widely used for studying interaction of various receptors and ligands. It is well known that immunoglobulin fusion protein of classic costimulatory molecules such as CD80 and CD86 could augment proliferation and cytokine production by T-cells in vitro. In addition, it was recently reported that CD80-Ig and CD86-Ig immobilized on the tumor surface can result in the cure of established tumors ***(8)***. Fusion proteins can also be used for blocking of the immune responses. For example, injection of CTLA-4-Ig, which binds CD80/CD86 and prevents interaction with CD28, prevents development of autoimmune diabetes ***(9)*** and prolongs allografts survival ***(10)***.

Materials

2.1. Cloning of Recombinant Gene

1. Sterile H_2O.
2. Human dendritic cells cDNA library.
3. Expression vector that has been modified to contain the sequence-encoding Fc portion of mouse IgG2a (e.g., pMIgV).
4. 60 m*M* $CaCl_2$.
5. Restriction enzymes and restriction enzyme buffer (New England Biolabs).
6. T4 DNA ligase and T4 DNA ligase buffer (New England Biolabs).
7. 6X DNA loading buffer (Promega).
8. Low-melting-point agarose (FMC).
9. Ethidium bromide (BioLogical).

2.2. Mammalian Cells Transfection

1. 293 cell line: adenovirus-transformed kidney epithelial cells (American Type Culture Collection).
2. Dulbecco's modified essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 2 m*M* L-glutamine, 100 U/mL streptomycin, 100 µg/mL penicillin.

3. 2.5*M* $CaCl_2$.
4. 2X HEPES buffer: 280 m*M* NaCl, 50 m*M* HEPES.
5. 150-mm tissue culture plates (Becton Dickinson).

2.3. Enzyme-Linked Immunosorbent Assay (ELISA)

1. Immulon 4 ELISA plates (Dynex).
2. Anti-mouse IgG mAb (Sigma).
3. Biotinylated anti-mouse IgG2a mAb (Pharmingen).
4. Horseradish-peroxidase-conjugated streptavidin (Caltag Laboratories).
5. Mouse IgG2a (Pharmingen).
6. Coating Buffer: 0.1*M* Na_2HPO_4, pH 9.0.
7. Phosphate-buffered solution (PBS): 8.5 g NaCl, 1.07 g Na_2HPO_4, 0.39 g NaH_2PO_4, in 1 L of deionized water, pH 7.4.
8. Blocking solution: PBS + 10% FBS.
9. Washing buffer: PBS + 0.005% Tween-20.
10. TMB substrate solution (Sigma).
11. Stopping solution: 0.5*M* H_2SO_4.
12. ELISA reader.

2.4. Purification of Fusion Protein

1. Prepacked Protein-G affinity column 1 mL volume (Pharmacia Biotech).
2. Start buffer: 20 m*M* Na_2HPO_4, pH 7.0.
3. Elution buffer: 0.1*M* glycine–HCl, pH 2.7.
4. 20% ethanol.

2.5. Cell Proliferation Assay Using Latex Microspheres

1. 5-µm latex microspheres (Interfastial dynamics).
2. Human T-cells.
3. Anti-human CD3 mAb (Pharmingen).
4. RPMI-1640 supplemented with 10% heat-inactivated FBS, 2 m*M* L-glutamine, 100 U/mL streptomycin, 100 µg/mL penicillin, and 5×10^{-5}*M* 2-mercaptoethanol.
5. PBS.
6. Blocking solution: PBS + 10% FBS.
7. ^{3}H-Thymidine.
8. 96-well flat-bottom plates.
9. β-counter.

3. Methods

3.1. Fusion Gene Vector Construction

The expression vector used for construction of the fusion protein should have the following basic elements:

- Promoter (e.g., CMV, SV40) for RNA polymerase binding and transcription initiation.
- Multiple cloning site (MCS) that contains restriction sites sequences recognized

by specific endonucleases for insertion of foreign DNA.
- Selection markers providing resistance to a antibiotics (e.g., neomycin, histidinol D).
- Elements necessary for expression of vector in *E. coli*.
- Sequence encoding a tag protein to which the gene of interest will be ligated (in our cases, the Fc portion of mouse IgG2a).

The exact protocol for designing and construction of the gene-encoding fusion protein depends on specific gene sequences. Here, we describe the most common steps that are involved in the gene fusion **(Fig. 1)**.

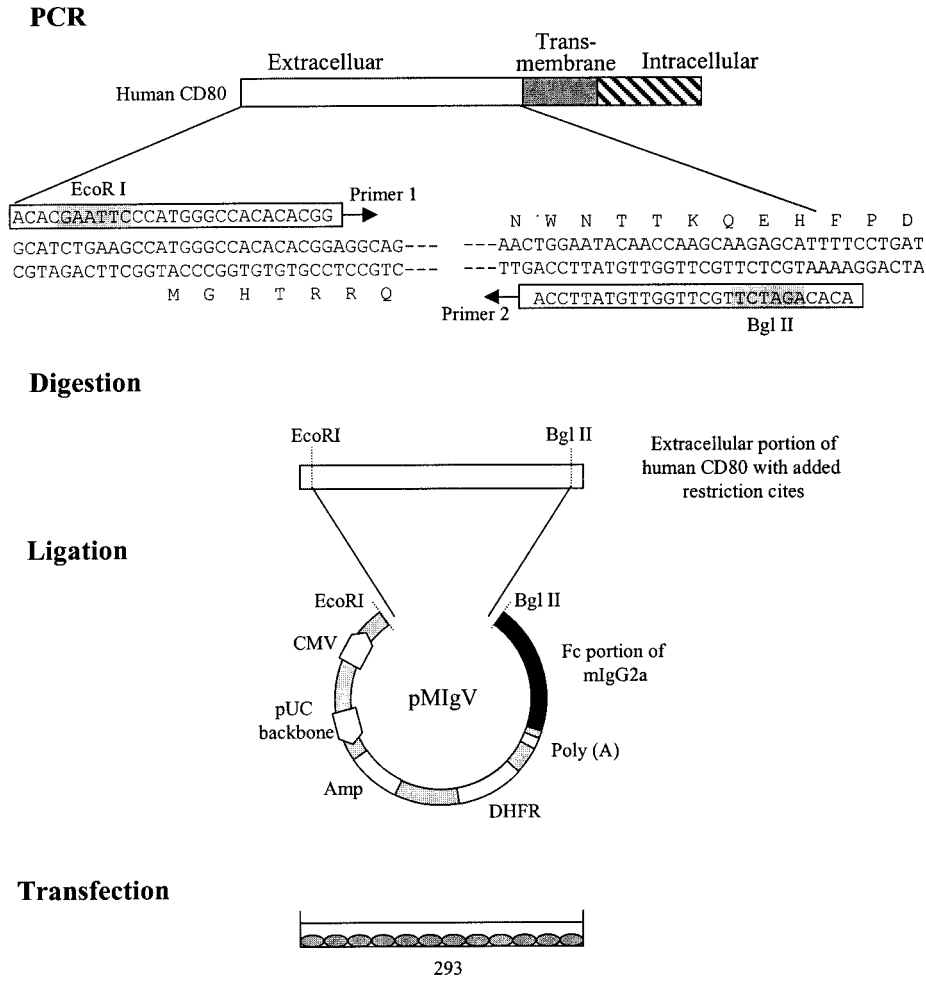
The first step is PCR amplification of a fragment of the gene-encoding extracellular portion of the desired costimulatory ligand (*see* **Note 1**). The PCR primers should be designed to incorporate new unique restriction sites for cloning the DNA-encoding extracellular portion of the molecule into a vector containing compatible restriction sites and tag protein-encoding sequence **(Fig.1)**. The amplified DNA-encoding extracellular portion of costimulatory ligand should be purified, digested by appropriate restriction enzymes, and then ligated into the vector.

DNA Digestion with Restriction Enzymes

1. Pipet the following into the microcentrifuge tube:
   1 µg DNA (plasmid or PCR product)
   2.5 µL 10X restriction buffer
   1 µL of restriction enzyme A
   1 µL of restriction enzyme B
   Bring volume to 25 mL using sterile H_2O.
2. Incubate the reaction mix at 37°C for 1 h to overnight.
3. Add DNA loading buffer and load all digested DNA onto 1% low-melting point (LMP) agarose gel containing ethydium bromide.
4. Electrophorese at 4°C and cut out the DNA under ultraviolet (UV) light.

Ligation

1. Melt LMP slices containing digested DNA (PCR product and vector) at 65°C
2. Mix 2 µL of vector LMP with and 8 µL insert LMP. Add 2 µL of 10X T4 ligation buffer and 7 µL of sterile H_2O, and keep the mixture at 37°C.
3. Add 1 µL of T4 DNA ligase.
4. Incubate overnight at 4°C.
5. Dilute ligation misture 1 : 5 in 60 m*M* $CaCl_2$.
6. Transform 10–20 µL of the diluted ligation mixture into competent *E. coli* (e.g., XL1-Blue).
7. Plate transformed *E. coli* on LBamp plates and culture overnight.
8. Select the positive colonies by PCR or restriction enzyme digestion.
9. Verify DNA sequence from several positive colonies (*see* **Notes 2** and **3**).
10. Expend one colony containing the correct recombinant DNA sequence and purify the plasmid DNA.



Fig, 1. Scheme for production and analysis of costimulatory molecule–Fc fusion protein. The extracelluar portion of most known costimulatory molecules can be obtained by polymerase chain reaction (PCR) from the cDNA library of dendritic cells (human CD80 molecule is taken as an example). During the PCR, two restriction sites (*Eco*RI and *Bgl*II, shaded sequence in the primers) are incorporated into DNA encoding the extracellular portion of human CD80. Digested PCR product is ligated into a vector containing The Fc portion of mouse Ig. The resulting vector is transfected into tumor cells. Fusion proteins can be detected and purified from supernatants of transfected cells.

3.2. Transfection of 293 Cells by Calcium Phosphate Precipitation Method

Calcium phosphate coprecipitation can be used for both transient and stable expression genes of interest in mammalian cells. For preliminary experiments, transient expression of the fusion gene is suitable. However, when higher amounts of fusion protein are required, the establishment of a permanent-cell line producing the protein might be considered.

1. Plate (1.0–2.0)X10^6 293 cells on a 150-mm tissue culture dish in 20 μL DMEM supplemented with 10% FBS 24 h before transfection.
2. On the day of transfection combine 20 μg of DNA with 124 μL of 2*M* $CaCl_2$ and bring the volume to 1 mL by with H_2O.
3. Add 1 mL of the 2X HEPES solution dropwise (*see* **Note 4**). Mix well and allow the tube to stand undisturbed for 10–20 min. The precipitate will be visible as an opalescent haze in the solution.
4. Add the calcium phosphate–DNA mixture directly to cells, carefully swirl the plate.
5. Incubate the cells overnight at 37°C in 8% CO_2.
6. The next day replace the medium with 10 mL of DMEM without FBS.
7. Collect supernatants 48–72 h posttransfection.

3.3. Detection of Fusion Protein in Cultural Supernatants

Because fusion protein described here contains the Fc portion of mouse IgG2a, it is possible to check the presence of the protein in supernatant of transfected cells by specific anti-mouse IgG2a ELISA.

1. Dilute anti-mouse IgG mAb in coating buffer at 2 μg/mL. Add 50 μL of this solution per well of a 96-well ELISA plate. Incubate over night at 4°C.
2. Empty the wells and add 100 μL of blocking buffer. Incubate for 30 min at room temperature.
3. Wash plate three times with washing buffer.
4. Add 50 μL/well serial dilutions in blocking buffer (1 : 2) of standard protein (mouse IgG2a) (*see* **Note 5**) and culture supernatants. Incubate plate for 60 min at room temperature.
5. Wash plate three times with washing buffer.
6. Add 50 μL/well of biotinylated anti-mouse IgG2a diulted at 1 μg/μL in blocking buffer. Incubate plate for 60 min at room temperature.
7. Wash plate three times with washing buffer.
8. Add 50 μL/well HRP–streptavidin conjugate diluted 1 : 3000 in blocking buffer. Incubate plates for 30 min at room temperature.
9. Wash plate six times with washing buffer.
10. Add 50 μL of TMB substrate solution. Incubate at room temperature for 5–20 min for color developing.
11. Stop color reaction by adding 50 μL/well of stopping solution. Color will change from blue to yellow.

12. Read the optical density using ELISA plate reader set to 450 nm.

3.4. Purification of Fusion Protein

One of the major benefits of fusion proteins is that they retain properties of both the molecule of interest and the tag molecule (mouse IgG2a in our case). Taking advantage of this feature, the immunoglobulin fusion protein can be purified from supernatants by Protein-G affinity chromatography. Prepacked ready-to-use Protein-G affinity columns are commercially available. Usually, transient gene expression does not give very high yield of fusion protein, because of this small volume, columns (1 mL) can be used.

1. Connect a 10cc syringe to 1 mL Protein-G column.
2. Add 3 mL of start buffer to syringe and push it through the column to wash out ethanol.
3. Push through additional 2 mL of start buffer to equilibrate column.
4. Apply the supernatant containing the fusion protein by pushing it through the column at a slow flow speed (approx 1 drop/s) (*see* **Note 6**).
5. Wash column with 5–6 mL of start buffer.
6 Elute the fusion protein with 3 mL of elution buffer. Collect 0.5-mL fractions.
7. Measure protein concentration in each fraction and combine fractions with highest protein concentration. Concentrate combined fractions and dialyze against PBS.
8 Wash the column with 3 mL start buffer and store in 20% ethanol at 4°C.

3.5. Proliferation Assay

One of the most informative and simple methods for the evaluation of costimulatory activity of the molecule is to measure its effect on the proliferation of T-lymphocytes. The proliferation of T-cells can be induce by immobilized anti-CD3 mAb, which directly crosslink the T-cell receptor complex. At low doses (40–200 ng/mL), immobilized antihuman CD3 mAb do not induce significant proliferation of T-cells, but in the presence of costimulatory molecules (e.g., anti-CD28 mAb, CD80-Ig, CD86-Ig), the proliferation is notably increased. Anti-CD3 mAb can be immobilized on any plastic surface, including tissue culture plates or recently described latex microspheres ***(11)***. Latex microspheres have certain advantages in activating T-lymphocytes and assaying costimulatory molecules. First, microspheres are easy to handle and once they are coated with mAb and/or fusion protein, they can be stored for months at 4°C without losing activity. The density of immobilized proteins on the microspheres can be easily varied and evaluated by FACS analysis. Because of their shapes, microspheres mimic natural cell–cell interaction, which is important in studying costimulation.

Coating Latex Microspheres

1. Place latex microspheres in a microcentrifuge tube at 5×10^7/mL in sterile PBS.
2. Add anti-human CD3 mAb alone or with fusion protein (*see* **Note 7**).

3. Incubate tube overnight at 37°C constantly shacking.
4. Wash microspheres two times in PBS by spinning at 400*g*.
5. Carefully discard the supernatant, trying not to disturb pelleted microspheres.
6. Resuspend the pellets in 1 mL PBS containing 10% FBS. Incubate tubes for 30 min at room temperature to block unbound sites of microspheres.
7. Count microspheres using a hemacytometer and store at 4°C until use.

Proliferation Assay

1. Distribute anti-CD3/fusion protein-coated latex microspheres 3×10^5 per well in 100 mL of supplemented RPMI-1640 in a 96-well flat-bottom plate.
2. Add human T-lymphocytes 3×10^5 cells/well in 100 mL supplemented RPMI-1640 (*see* **Note 8**).
3. Incubate cells for 48 h at 37°C 5%CO_2. Then, add 1 µCi/well of ^{3}H-thymidine solution.
4. After 24 h incubation at 37°C, harvest the cells and determine incorporated ^{3}H-thymidine in a β-counter.
5. Compare ^{3}H-thymidine incorporation in wells containing microspheres coated with anti-CD3 alone and coated with anti-CD3 plus costimulatory molecule.

4. Notes

1. The most reliable source known for cloning and probably novel costimulatory ligands is dendritic cells (DC) cDNA library, as DC are professional antigen-presenting cells expressing many different accessory molecules on their surface.
2. High-fidelity DNA polymerase should be use to reduce the mutation rate.
3. After ligation of the PCR product to the vector, DNA sequencing is always required to verify whether the junction of two DNAs encoding the extracellular portion of the ligand and Fc are in frame.
4. Make sure that the pH of the HEPES solution is 7.2; this is an absolutely critical parameter.
5. The starting concentration of the standard for ELISA should be about 20–50 ng/mL.
6. It is very difficult to push a large amount of supernatant through the column. We recommend concentrating the supernatant before applying it to the column.
7. Although the concentration of 0.2–0.5 µg/mL of anti-human CD3 mAb usually result in sufficient T-cell proliferation, it is useful to determine the optimal anti-CD3 dose for your conditions in initial pilot experiments. The same is applied to fusion proteins. In general, for the best costimulation, microspheres are coated with 2.5–10 µg/mL of fusion proteins. If you use various concentrations of mAb and/or fusion proteins for coating, it is important to keep the same final concentration of total protein by adding control IgG.
8. The population of T-cells enriched from human peripheral mononuclear cells by nylon wool is quite suitable for activation by latex microspheres coated with anti-CD3 and costimulatory molecules.

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Adenovirus-Mediated Drug-Sensitivity Gene Therapy for Hepatocellular Carcinoma

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1. Introduction

Selective gene therapy represents a potent approach in cancer treatment that utilizes a cell's own nontoxic suicide genes. Currently, the suicide genes under investigation mediate sensitivity by encoding viral or bacterial enzymes that convert inactive prodrug into toxic antimetabolites that inhibit nucleic acid synthesis ***(1,2)***.

Perhaps one of the most intensely studied suicide genes is the herpes simplex virus type-1 thymidine kinase (HSV-tk) **(Fig. 1)**. Although mammalian tk specifically phosphorylates thymidine to generate thymidine monophosphate (dTMP), HSV-tk can convert the antiviral nucleoside analogs acyclovir (ACV), ganciclovir (GCV), and bromovinyldeoxyuridine (BVdU) to their corresponding nucleotide monophosphate derivatives. The monophosphate forms of ACV and GCV, and perhaps, to a small extent, BVdU are subsequently phosphorylated by endogenous cellular kinases to triphosphates. These molecules are potent inhibitors of DNA polymerases, leading to the disruption of cellular DNA synthesis and ultimately cell death.

Another suicide gene therapy that has recently received considerable attention is cytosine deaminase (CD), which is an enzyme present in some bacteria and fungi but absent in animal cells **(Fig. 1)**. Its function is to catalyze the deamination of cytosine to uracil and also the deamination of the nontoxic analog 5-FC to the active antimetabolite 5-fluorouracil (5-FU). 5-FU, although a widely used chemotherapuetic agent, has a limited effect in the treatment of

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

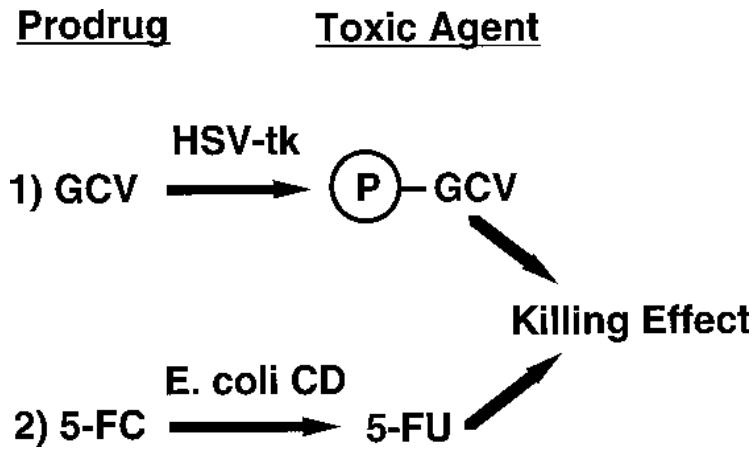

Fig. 1. Suicide genes and prodrug.

human solid tumors because of its resistance to the cytotoxic effects of 5-FU ***(3)***. *Escherichia coli* uracil phosphoribosyltransferase (UPRT) is a pyrimidine salvage enzyme that catalyzes the synthesis of uridine monophosphate from uracil and 5-phosphoribosyl-α-1-diphosphate. It was reported that adenovirus-mediated transduction of *E. coli* UPRT gene results in marked sensitization of cancer cells to a low concentration of 5-FU. The adenovirus vector transduction of the uracil phosphoribosyltransferase gene followed by 5-FU administration may be representative of a new chemosensitization strategy for cancer gene therapy ***(4)***.

The bystander effect has proven to be valuable in gene therapy. The bystander effect is where the active chemotherapeutic agent diffuses from the tumor cell in which it was produced to the neighboring malignant cells in sufficient concentrations to suppress growth. There are several mechanisms by which the bystander effect can be elicited, the first mechanism utilizes gap junctions or simple diffusion for transfer of toxic metabolic products, the second mechanisms is through phagocytosis of apoptotic vesicles of dead tumor cells by live tumor cells that mediate apoptosis and finally, induction of an immune response against the tumor. Currently there are several clinical trials underway to determine the efficacy of the bystander effect in human gene therapy.

Suicide genes must be introduced into cells in ways that ensure their uptake and expression in as many cancer cells as possible while limiting expression by normal cells. Suicide gene therapy for cancer demands that the vector itself has the capacity to discriminate between target and nontarget cells. The targeting strategy for transcriptional control subsequent to uncontrolled nonspecific gene transduction may implement the use of cellular cis-acting sequences ***(5)***. This strategy may

remain as a safety feature even in the event of the development of reliable transductionally targeted vectors. A second technique that is designed to avoid this problem is the use of vectors that infect or transduce only target cells. The adenovirus vector has numerous attractive features for this development ***(2)***. It is hopeful that the combination of adenovirus-mediated tumor-specific suicide gene expression and prodrug treatment will provide a new therapeutic strategy for cancer gene therapy.

2. Materials

2.1. Recombinant Replication-Deficient Adenoviruses (Fig. 2)

1. AdAFPtk; adenovirus vector containing the HSV-tk gene driven by α-fetoprotein (AFP) enhancer/promoter.
2. AdAFPCD; adenovirus vector containing *E. coli* cytosine deaminase gene driven by AFP enhancer/promoter.
3. AdAFPlacZ; adenovirus vector containing the nuclear localization signal (nls) tagged *E. coli* β-galactosidase (*lacZ*) gene (nls-*lacZ*) driven by AFP enhancer/promoter.
4. AdCAtk; adenovirus vector containing the HSV-tk gene driven by nonspecific and strong CAG promoter (composed of a cytomegalovirus immediate–early enhancer and modified chicken β-actin promoter).
5. AdCACD; adenovirus vector containing the CD gene driven by the CAG promoter
6. AdCAlacZ; adenovirus vector containing the nls-*lacZ* gene driven by the CAG promoter

2.2. Cell Culture

Human hepatocellular carcinoma (HCC) HepG2 and PLC/PRF/5 and human embryonal kidney 293 cell lines are available from American Type Culture Collection (Rockville, MD). Human HCC cell lines of HuH-7 and HLF were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). HuH-7 cells were cultured in RPMI-1640 medium containing 0.5% fetal bovine serum (FBS) and 0.2% lactalbumin hydrolysate and HepG2 and PLC/PRF/5 cells were cultured in DMEM with 10% FBS. HLF cells were grown in modified essential medium (MEM) with 10% FBS and 293 cells were maintained in Dulbecco's MEM (DMEM) with 5% FBS. HepG2, PLC/PRF/5, and HuH-7 cells produce AFP, whereas HLF cells do not produce AFP. The 293 cells grow as an attached monolayer and can be detached using a solution of phosphate buffered saline (PBS) containing 0.5 m*M* EDTA. Cultures were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2.

2.3. lacZ Staining

1. Phosphate-buffered saline (PBS)
2. Cell fixation solution: 0.5% glutaraldehyde/PBS; make fresh each time.

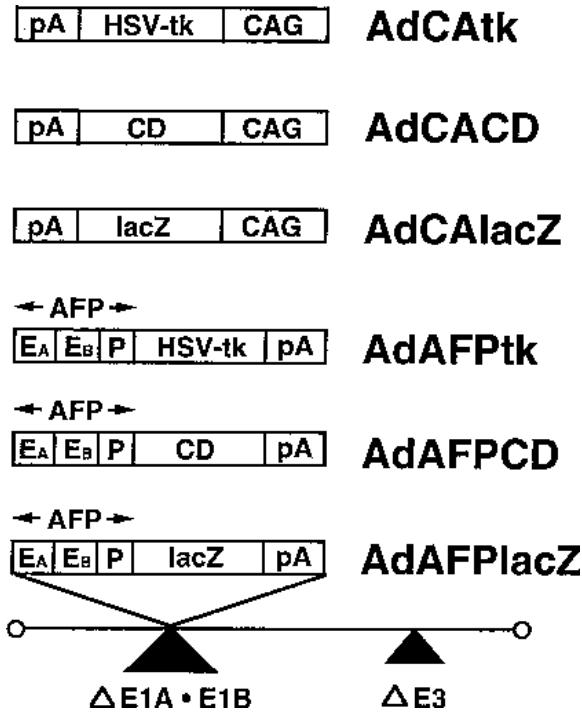

Fig. 2. Replication-deficient adenoviruses. A recombinant replication-defective adenovirus lacking the viral E1 and E3 region was used to construct these adenovirus vectors. HSV-tk, herpes simplex virus thymidine kinase; CD, *E. coli* cytosine deaminase gene; *lacZ*, *E. coli* β-galactosidase gene; EA and EB, 728-bp fragment covering the α-fetoprotein (AFP)-enhancer domain A and B (–4.0 to –3.3 kb); P, 0.17-kb AFP promoter; pA, the SV40 early mRNA polyadenylation signal. CAG promoter is composed of the cytomegalovirus enhancer and the modified β-actin promoter ***(6)***. AdCAtk, adenovirus containing HSV-tk driven by CAG promoter; AdCACD, adenovirus containing CD driven by CAG promoter; AdCAlacZ, adenovirus containing the *lacZ* gene driven by CAG promoter; AdAFPtk, adenovirus containing HSVtk gene driven by AFP promoter/enhancer; AdAFPCD, adenovirus containing CD driven by AFP promoter/enhancer; AdAFPlacZ, adenovirus containing the nuclear localized *lacZ* gene driven by AFP promoter/enhancer.

3. Tumor xenograft fixation solution: 4% paraformaldehyde–PBS. Add 8 g paraformaldehyde in 200 mL PBS. Stir at 60–80°C and add several drops of 1*N* NaOH until the solution becomes transparent. Store it in brown bottle at 4°C. Use within a week.
4. 1*M* $MgCl_2$ (1000X stock solution).
5. 40 mg/mL X-gal (40X stock solution): Dissolve 400 mg 5-bromo-4-chloro-3-

indolyl-β-D-galactopyranoside (X-gal) in 10 mL *N*,*N*-dimethylformamide. Store in aliquots at –20°C.
6. 0.1*M* KFeCN (20X stock solution): 0.1*M* $K_3Fe(CN)_6$ and 0.1*M* $K_4Fe(CN)_6$ in H_2O. This solution may be stored at 4°C.
7. X-gal staining solution: Mix 0.5 mL of 40 mg/mL X-gal (40X), 1.0 mL of 0.1*M* KFeCN (20X), 20 µL of 1*M* $MgCl_2$ (1000X), 18.5 mL of PBS (total 20 mL), make fresh each time.
8. Eosin staining solution.
9. Microscope.

2.4. Cytosine Deaminase Enzymatic Activity

1. Cell lysis buffer: 100 m*M* Tris–HCl pH 7.8 and 1 m*M* EDTA.
2. [6-^{3}H]cytosine (0.14 mCi/mmol, Moravek Biochemicals).
3. 30 m*M* cytosine stock: Dissolve cytosine in H_2O; store at –20°C.
4. 1*M* acetic acid.
5. SCX Bond Elute (Varian).
6. ACS II (aqueous counting scintillant) (Amersham).
7. Scintillation counter.

2.5. In Vitro Killing Assay

1. 50 mg/mL GCV stock solution: Dissolve 500 mg of ganciclovir (GCV) (Syntex Laboratories, Inc., Palo Alto, CA) in autoclaved H_2O. Stock solution may be stored for at least 1 mo at 4°C. Prior to assay, dilute stock solution to the desired final concentration in appropriate medium.
2. 0.5 mg/mL MTT solution: Dissolve 0.25 g of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Lous, MO) in 50 mL PBS.
3. 100 m*M* 5-fluorocytosine (5FC) stock solution: 0.645 g 5FC per 50 mL saline. Filter sterilize and store in aliquots at 4°C. Prior to assay, dilute stock solution to the desired final concentration in appropriate medium.
4. 50 mg/mL 5FU solution (Roche, Switzerland).
5. Falcon Cell Culture Inserts with a pore size of 0.45 µm (Becton Dickinson and Co., Franklin Park, NJ).
6. 24-well plates (Becton Dickinson and Co., Franklin Park, NJ).
7. Ethanol.
8. Automatic plate reader.
9. Coulter Counter.

2.6. In Vivo Treatment

1. Athymic BALB/cA *nu/nu* mice (8 wk old).
2. 1-mL syringe.
3. 30-gage needle.
4. Saline.
5. Calipers.

6. 10 mg/mL 5FC stock solution. 1 g 5FC/100 mL saline. Filter sterilize and store in aliquots at 4°C.

Note: All chemicals should be of analytical grade.

3. Methods

3.1. Adenovirus Production

For a more complete description of the production of the adenovirus, see Chapter 3. Construction of recombinant adenovirus vectors **(Fig. 2)** were performed by homologous recombination between the expression cosmid and the parental virus genome as described ***(7–10)***. The recombinant adenoviruses were propagated with 293 cells, and viral solution was stored at –80°C. The titers of viral stocks (PFU: plaque-forming unit) were determined by plaque assay on 293 cells. Prior to assay, thaw adenovirus stock solution quickly in water bath and dilute to the desired final concentration with appropriate medium. Viral stocks used for experiments do not contain detectable replication-competent viruses as evaluated using the PCR assay, which requires two pairs of primers in the same reaction to detect adenoviral E1A DNA with coamplification of E2B DNA as an internal control ***(11)***.

3.2. Transduction of LacZ Gene by Adenovirus In Vitro

Prior to conducting the killing assay in vitro, it is necessary to determine the efficiency of adenovirus-mediated gene transduction and the specificity of the promoter.

1. Plate exponentially growing hepatoma cells at a density about 5×10^5 cells/well in duplicate six-well culture plates 24 h before infection.
2. Count the cell number of one of the duplicate wells. Dilute original adenovirus stock solution with culture medium. Aspirate medium and add 1 mL of various amounts of adenovirus solution onto the cell monolayers, distribute over the monolayer, and incubate cells at 37°C. The ratio of the number of adenovirus to a cell is expressed as a multiplicity of infection (MOI).
3. After 24 h, stain cells with X-gal to demonstrate *lacZ* gene product as follows. Rinse cells with PBS twice and fix in PBS containing 0.5% glutaraldehyde for 10 min at room temperature. Rinse cells with PBS containing 1 m*M* $MgCl_2$ three times and allow them to react in X-gal staining solution for 3–12 h at 37°C.
4. Rinse cells with PBS containing 1 m*M* $MgCl_2$ three times and count and photograph for β-gal positive cells. Score 100 cells of four independent fields of view **(Fig. 3)** (*see* **Note 1**).

3.3. Enzymatic Activity of Transduced Gene

The method described as follows is for the analysis of the enzymatic activity of *E. coli* cytosine deaminase. Enzymatic assay for HSV-tk is described elsewhere ***(11,12)***.

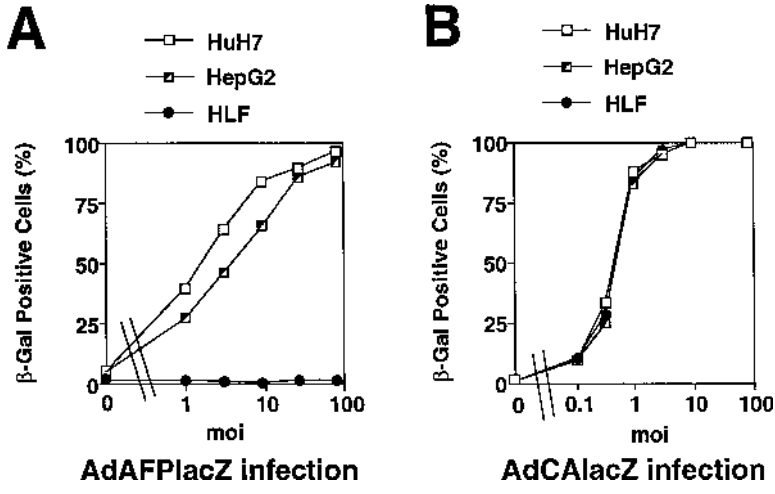

Fig. 3. Gene transduction efficiency of adenovirus in vitro. Cells were plated in six-well plates and infected with (A) AdAFPlacZ or (B) AdCAlacZ at different MOIs ranging from 0 to 3^4; 24 h later, cells were fixed and stained with X-gal to demonstrate *lacZ* expression. Values are represented as mean of β–gal-positive cells from scoring 100 cells of four independent fields of view. (Data from **ref. *9***, reprinted with permission.)

1. Aspirate medium from subconfluent hepatoma cells in a 100-mm dish and add 1 mL of CD or *lacZ*- expressing (negative control) adenoviruses at a MOI of 3 PFU/cell to the dish (*see* **Note 2**).
2. Incubate the plate at 37°C for 60 min. Swirl the plate every 20 min.
3. After 60 min, add 4 mL of growth medium to the plate and incubate at 37°C.
4. After 24 h, wash cells three times with cold PBS, then resuspend in 1 mL of cold cell lysis buffer. Prepare soluble cell extracts by sonication followed by centrifugation to remove cellular debris and determine the protein concentration. All procedure should be done on ice.
5. Add 10 µg of cell extract to an Eppendorf tube on ice and adjust volume to 13.4 µL with cell lysis buffer.
6. Add 1.5 µL of 30 m*M* cytosine and 0.1 µL [6-^{3}H]cytosine (0.14 mCi/mmol) to the tube (final volume is 15 µL). Mix and incubate at 37°C for 60 min.
7. Add 345 µL of 1*M* acetic acid to stop reaction.
8. Separate the reaction product [^{3}H]uracil from [^{3}H]cytosine using SCX Bond Elute (Varian) as follows. Load sample to column that had been previously rinsed with 1 mL of 1*M* acetic acid. Centrifuge samples in 15-mL disposable tubes (1000 g for 1 min at room temperature) and collect the follow-through.
9. Apply 0.5 mL wash volume of 1*M* acetic acid to the column, centrifuge (1000 *g* for 1 min at room temperature) and combine the eluent with the first follow-through (containing [^{3}H]uracil).
10. Add 5 mL of ACS II (aqueous counting scintillant), mix and count the radioactivity with scintillation counter (*see* **Note 3**).

3.4. Killing Assay In Vitro

3.4.1. GCV Sensitivity of HSV-tk-Expressing Adenovirus-Infected HCC Cell Lines

1. Plate hepatoma cells at a density of 5×10^3 cells/well in 96-well plates 24 h before infection.
2. Put adenovirus (AdAFPtk, AdAFPlacZ, AdCAtk, and AdCAlacZ)-containing medium onto cells at a MOI of 3 PFU/cell (*see* **Note 4**). The total volume of the adenovirus solution is 50 µL/well.
3. After incubation for another 24 h, aspirate the medium and replace with fresh medium containing various concentrations of GCV. Renew the medium containing GCV on d 3. Culture cells at 37°C for 6 d.
4. Measure the number of cells by a colorimetric assay as follows. Aspirate medium and add 100 µL of 0.5 mg/mL of MTT in PBS to each well and culture cells for 1–3 h at 37°C. Aspirate supernatant, add 100 µL of 99.5% ethanol to each well and mix. Quantitate the color reaction using an automatic plate reader at 570 nm with a reference filter of 630 nm **(Fig. 4)** (*see* **Note 5**).

3.4.2. 5FC Sensitivity of E. coli CD-Expressing Adenovirus-Infected HCC Cell Lines

The protocol is essentially the same as described in **Subheading 3.4.1.**, however, the sensitivity of the cells to 5FU also should be determined.

1. Plate cells at a density of 5×10^3 cells/well in 96-well plates.
2. The next day, aspirate the medium from wells and put 50 µL of adenoviruses (AdAFPlacZ, AdAFPCD, AdCAlacZ, and AdCACD) onto hepatoma cells at a MOI of 3 PFU/cell.
2. After incubation for a subsequent 24 h, aspirate the medium and replace with new medium containing various concentrations of 5FC. Also, make dilutions of 5FU with medium and start culturing cells with various concentrations of 5FU (*see* **Note 6**).
3. Culture cells at 37°C for 6 d. Renew medium on alternate days. Measure the number of viable cells with the MTT assay.

3.5. Bystander Effect

Currently available vectors for gene therapy are not capable of transferring a gene to all tumor cells; however, successful application of suicide gene therapy relies on the bystander effect.

3.5.1. Analysis of Cell–Cell Contact Dependent Bystander Effect

The method described as follows is for the analysis of the bystander effect in the HSV-tk/GCV system in vitro.

1. Infect subconfluent cells in a 100-mm tissue culture dish with adenovirus vector expressing HSV-tk at a MOI of 3.

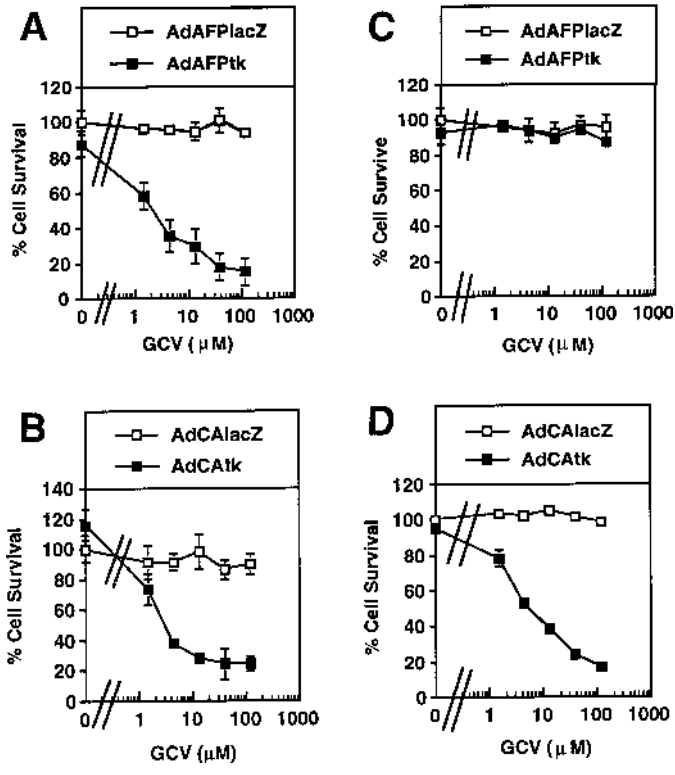

Fig. 4. Comparison of GCV dose response in human hepatoma cell lines (A,C) HuH-7 and (B,D) HLF infected with tk-expressing adenoviruses. Cells in a 96-well plate were infected with AdAFPtk or AdCAtk at a MOI of 3 and cultured in the presence of various concentration of GCV for 6 d. MTT assay was used to measure cell numbers. The points on the ordinate correspond to the percentage of surviving cells compared to AdAFPlacZ- or AdCAlacZ-infected cells without GCV as 100%. Adenovirus encoding tk-infected cells are compared to cells infected with *lacZ*-expressing adenovirus. Data are shown as mean ± SD. (Data from **ref. *9,*** reprinted with permission)

2. After 24 h, rinse cells extensively three times with medium to remove uninfected viruses, trypsinize, and mix with various number of uninfected cells in varying ratios and plate into 96-well plates at a density of 1×10^4 cells /well to make cell–cell contact (*see* **Note 7**).
3. Incubate cells at 37°C in the presence of 20 μ*M* of GCV (*see* **Note 8**) for 6 d and measure the number of cells by the MTT assay.

3.5.2. Analysis of Bystander Effect by the Two-Chamber System

To determine whether direct cell–cell contact was necessary for the bystander effect, culture inserts with the pore size of 0.45 μm could be used to

prevent direct cellular contact between suicide-gene-expressing adenovirus-infected and uninfected cells. The bystander effect of the CD/5FC system is also called the neighbor cell killing effect.

1. Infect cells with AdAFP-CD or AdAFP-*lacZ* at a MOI of 3 in six-well plates. Meanwhile, the uninfected cells were seeded into 24-well plates (Becton Dickinson and Co., Franklin Park, NJ) at a density of 1×10^5 cells/well.
2. After 24 h, rinse the AdAFP-CD or AdAFP-*lac*Z infected cells extensively with medium twice to remove the free virions, trypsinize, and count by Trypan Blue exclusion, then plate into Falcon Cell Culture Inserts at various ratios to the uninfected cells (0.01–1.0).
3. Consequently, form a double-chamber system by putting the Falcon Cell Culture Inserts (upper chamber) containing AdAFP-CD or AdAFP-*lacZ* infected cells into the 24-well plates (lower chamber) where uninfected cells were plated.
4. Coculture infected and uninfected cells in 500 μ*M* of 5FC-containing media. After 6 d, measure the number of viable uninfected cells by MTT assay (*see* **Note 9**).

3.6. Animal Experiment

3.6.1. Gene Transduction Efficiency by Adenovirus Vector In Vivo

To evaluate the ability of adenovirus-mediated gene transfer to hepatocellular carcinoma in vivo, establish tumor foci subcutaneously in nude mice, and stain with X-gal after direct injection of *lac*Z-expressing adenovirus into tumor.

1. Suspend 10^7 PLC/PRF/5 cells in 100 μL saline and inject subcutaneously into the flank of athymic BALB/cA *nu/nu* mice (8 wk old) (*see* **Note 10**).
2. Let tumors grow for 1–3 wk until the diameter of the tumor becomes about 5 mm.
3. Inject a total volume of 100 μL of *lacZ*-expressing adenoviruses (10^9 PFU) directly into the tumor from three directions using a 1-mL syringe and a 30-gage needle.
4. After 48 h, sacrifice the animals, remove tumors, cut into small pieces, and fix in 4% paraformaldehyde–PBS on ice for 3 h.
5. After washing with PBS, put tumors into X-gal staining solution and stain at 37°C for several hours (*see* **Note 11**).
6. After washing with PBS, embed in paraffin, microtome 5-mm sections, and counterstain with Eosin (*see* **Note 12**).

3.6.2. In Vivo Tumor Treatment

The method described as follows is for the analysis of the antitumor effect of AdAFPCD or AdCACD plus systemic 5FC administration in vivo.

1. Suspend 10^7 PLC/PRF/5 cells in 100 μL saline and inject subcutaneously into the flank of athymic BALB/cA *nu/nu* mice (8 wk old).
2. Let tumors grow for 1–3 wk until the diameter of the tumor become about 5 mm, in **Subheading 3.6.1.** Tumors are randomized in a blind manner to be directly transduced by injecting a total volume of 100 μL directly into the tumor from

three directions for three successive days using a 1-ml syringe and a 30-gage needle with the following: AdAFPCD (10^9 PFU) or AdCACD (10^9 PFU) subsequently with or without 5FC (500 mg/kg body weight intraperitoneally), or control vehicle (DMEM/10% FBS) with or without 5FC.
3. Inject 5FC (500 mg/kg) intraperitoneally daily for 2 wk. Measure tumor size using calipers up to d 35, and calculate the tumor volume as [length (mm) × width $(mm)^2$]/2.

4. Notes

1. If possible, use nuclear localized signal (nls) tagged *lacZ*, as it is easy to distinguish a true positive signal from a nonspecific cytosol background. Cells exhibiting characteristic nuclear/perinuclear blue, indicating β-gal, are considered positive for expression of the nls-tagged lacZ gene product. One can underestimate the number of cells expressing *lacZ* because there may be a lower threshold at which one can visualize expression. It is critical to do a negative control (i.e., empty vector, suicide-gene-expressing virus, or uninfected cells for *lacZ* staining). You can achieve 100% gene transduction efficiency by using 1–10 MOI of adenovirus for hepatoma cells **(Fig. 3)**. Gene transduction efficacy of HCC cell lines is approx 10 times higher than other gastrointestinal-cancer-derived cell lines.
2. To get 100% gene transduction efficiency, use a minimum amount of adenovirus, as too much adenovirus will damage cells.
3. The enzymatic activity of CD was calculated as the difference of the nonspecific radioactivity of parental cell lysates from the radioactivity of AdAFPCD- or AdCACD-infected cell lysates. The use of SAX Bond Elute column is widely used, and relatively simple to perform and, therefore, suitable for initial experiment. However, a good alternative may be separation by thinlayer chromatography ***(11)***.
4. Optimize the amount of the adenoviruses. Use minimum adenoviruses to give 100% gene transduction efficiency.
5. Optimize the MTT assay by determining the linear growth range over an increasing time frame by taking optical density reading at 570–630 nm. If these wavelengths are not available on plate readers, a suitable wavelength may be found empirically. Absorbance should be directly proportional to the number of cells. A factor that will effect the cytosine deaminase (CD) reading is cell number and, therefore, make an initial dilution of the cells onto two plates. Use one plate to count cells in a Coulter Counter and use the second plate for the MTT assay. The number of the cells for plating will also depend on cell types.
6. It is necessary to know that each cell line is sensitive to 5FU.
7. In order to get cell–cell contact, the number of cells plated will be dependent on the size of the cells. Phosphorylated GCV will migrate across the plasma membrane into the adjacent cell via gap junctions; thus, it is necessary for cells to form contacts with each other. However, with regard to the CD/5FC system, converted 5FU is able to cross the plasma membrane; therefore, contact between cells is not required.
8. Optimize the concentration of GCV based on the results of in vitro killing assay. In order to exclude the possibility of carryover of adenoviruses from the previous

culture dish, rinse cells extensively to remove uninfected viruses. In addition, confirm that the cytotoxicity of AFP-producing cells by GCV is not demonstrated by culturing the cells in the medium obtained from the mixture of AdAFPtk-infected and -uninfected cells.

9. To exclude the possibilities of the diffusion of virus and/or cytosine deaminase from the upper chamber could affect the viability of the uninfected cells at the lower chamber, a double-chamber system with CD-infected cells at the upper chambers at various ratios (0.01–1.0) to uninfected cells at the lower chambers was set and cultured in 5-FC free media for 2 d in advance. As control, another double-chamber system with similar condition except that AdlacZ, instead of AdCD, infected cells were plated at the upper chamber was designed. Then, the upper chambers, containing the AdAFP-CD or AdAFP-*lacZ* infected cells, were removed, and the uninfected cells in the lower chambers were further cultured in the presence of 500 μ*M* of 5-FC for an additional 6 d. The number of viable uninfected cells was then measured. Optimize the concentration of 5FC based on the results of in vitro killing assay.
10. The following HCC cell lines are subcutaneously transplantable into nude mice: HuH-7, PLC/PRF/5, HepG2, Hep3B, and HLF.
11. Staining of tumor cells with X-gal may require overnight incubation.
12. If adenoviruses are injected correctly, about 50% of the tumor cells express the reporter gene.

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V

Clinical Protocol for p53 Gene Therapy for Liver Tumors

20

Clinical Protocol for *p53* Gene Therapy for Liver Tumors

Michael D. Kelly, Ragai R. Mitry, and Nagy A. Habib

1. Introduction

This chapter is intended to help other workers with the preparation of human gene therapy proposals. What follows is an abridged version of a protocol describing the use of gene replacement with *p53* for liver tumors. This was submitted to the Gene Therapy Advisory Committee (GTAC) of the Department of Health (United Kingdom). This is the first trial to be approved by GTAC for gene therapy of liver tumors in humans.

2. Clinical Protocol

Abstract: The human *p53* gene is a tumor-suppressor gene involved in the control of cell proliferation. Loss of wild-type *p53* (wt *p53*) function is associated with the development of many types of human cancers. In vitro and in vivo studies have demonstrated that the reintroduction and expression of wt *p53* in tumor cells with *p53* mutations has been shown to suppress tumor growth or induce apoptosis in both in vitro and in vivo models ***(1)***.

This study seeks to evaluate the safety, biological efficacy, and the effectiveness of treatment with a recombinant replication-deficient adenoviral vector (wtp53–CMV–Ad) containing a sequence-encoding wt-p53 whose expression is under the control of the human cytomegalovirus (CMV) immediate–early promoter–enhancer. It will be administered by infusion via the hepatic artery for the regional gene therapy of malignant liver tumors. Study patients will have incurable liver tumors with evidence of *p53* alteration. The vector Ad-p53 is a recombinant adenovirus (serotype 5) grown in 293 cells, which contain the adenoviral E1A and E1B coding sequences that have been removed from the vector.

From: *Methods in Molecular Medicine, vol. 45: Hepatocellular Carcinoma Methods and Protocols*
Edited by: N. A. Habib

The study design is an open-label, nonrandomized, single-dose, dose-escalation/Phase I/II clinical trial anticipated to involve a maximum of 19 patients. The wtp53–CMV–Ad will be administered as a single bolus infusion via the hepatic artery for regional gene therapy in three escalating doses to successive cohorts of three patients each, until the maximum tolerated dose is determined. Subsequently, 10 patients will be treated with this dose. The route of administration of wtp53–CMV–Ad via hepatic artery infusion is designed to maximize gene therapy exposure to the malignant tumors while minimizing exposure to normal tissues outside the liver. The clinical protocol is designed to monitor treatment toxicity. Another objective is to evaluate the biological efficacy, including efficiency and stability of gene transfer by analysis of tumor tissues following therapy. As an important part of this objective the pharmacokinetics of wtp53–CMV–Ad will be studied. Clinical evidence of antitumor efficacy will also be collected. In addition, the safety and efficacy of different doses levels of wtp53–CMV–Ad will be studied.

2.1. Objectives

(a) To assess the safety of wtp53–CMV–Ad when given as a single hepatic artery bolus infusion.
(b) To assess the biological efficacy of wtp53–CMV–Ad in liver tumors when given as a hepatic artery bolus infusion. Biological efficacy, including efficiency and stability of gene transfer, will be studied by analysis of tumor tissue following therapy. Clinical evidence of antitumor efficacy will also be noted.
(c) To assess the effect of dose of wtp53–CMV–Ad given as a hepatic artery bolus infusion on safety and efficacy, wtp53–CMV–Ad will be given in escalating doses to successive groups of patients as tolerated. The effect of dose on patient tolerance and toxicity, as well as on biological and clinical efficacy, will be studied. As an important part of this objective, the pharmacokinetics of wtp53–CMV–Ad will be studied.

2.2. Rationale of the Proposed Research

The use of wtp53–CMV–Ad to treat malignant tumors of the liver constitutes a novel form of cancer treatment in which introduction of the wt-p53 tumor-suppressor gene into *p53*-altered tumors may inhibit tumor growth and thus have beneficial clinical impact. The rationale for the administration of wtp53–CMV–Ad via hepatic artery infusion is its potential to maximize wtp53–CMV–Ad exposure to liver tumors while minimizing exposure to normal tissues outside the liver.

3. Role of *p53* in Liver Tumors

The *p53* gene plays an important role in cell-cycle regulation and it is believed that loss of wild-type *p53* function directly promotes oncogenesis. In particular, alteration of the tumor-suppressor gene *p53*, via mutation and/or allelic loss, has been

demonstrated to be a critical event in colorectal tumorigenesis and approximately half of colorectal liver metastases show *p53* mutation ***(2)***.

3.1. Recombinant Adenoviral Vectors for In Vivo Gene Therapy

Live, wild-type (nonrecombinant) adenoviruses have been used clinically as vaccines for prophylaxis of adenoviral upper respiratory infection, a disease of low morbidity but high incidence. These vaccines, which were at one time given routinely to military recruits, are well tolerated and are considered nononcogenic. Recombinant versions of adenovirus have entered clinical trials, also as oral vaccines. Thus far, they too appear to be without significant toxicity. Recently, clinical trials using recombinant, replication-defective adenoviral vectors for gene therapy have been initiated. In these trials, recombinant adenoviral vectors carrying the gene for the cystic fibrosis transmembrane conductance regulator (CFTR) are given via intra-airway administration to patients with cystic fibrosis.

A Phase I study at the Gustave Roussy Institut was initiated to evaluate the feasibility, safety, and clinical effects of the intratumor administration of a recombinant-deficient adenovirus (Ad) containing the marker gene encoding the *E. coli* enzyme β-galactosidase (β-gal) (Ad–β-gal) in untreated patients with advanced lung cancer. The first dose level was 10^7, the second 10^8, and the third 10^9 PFU (three patients per level). All patients received concomitant chemotherapy. β-Gal express (X-gal staining) was observed in three out of six tumor biopsies. The microbiological and immunological follow-up of patients who were carriers of wild-type Ad before injection, showed that viral cultures of bronchoalveolar (BAL) specimens taken immediately after Ad–β-gal injection were positive in all patients. All body fluid specimens were positive at polymerase chain reaction (PCR) analysis within the first 10 d after injection, as were blood samples drawn 30 min after injection in the three patients at the second dose level. BAL samples remained positive at 1 mo in two patients and at 3 mo in one patient after Ad–β-gal injection. No antibody (Ab) response to β-gal was noted in patients, but four had a significant rise in their anti-Ad Ab titers. All 363 samples (throat and stools) taken from 54 staff before and after injection of patients were negative for wild-type Adv and Ad–β-gal. Sera tests (CF) in 202 medical staff were also negative for anti-Adv Ab titers. This study shows that a marker gene can be safely transferred into human tumor cells with a recombinant Adv vector ***(3)***.

4. Design of the Research

4.1. Structure and Characteristics of the System of Gene Transfer

A novel antineoplastic agent, wtp53–CMV–Ad, consists of a recombinant adenoviral vector containing the cloned human wild-type *p53* tumor-suppres-

sor gene. The adenoviral vector is derived from adenovirus type 5, a common serotype belonging to subgroup C. Our vector has been rendered replication defective through deletion of region E1, which contained the viral genes E1A, E1Bm, and protein IX. It is propagated in the human embryonal kidney cell line 293, which contains adenoviral sequences to support replication of the otherwise replication-defective vector. A cDNA sequence encoding the entire open-reading frame of the human wild-type *p53* gene has been inserted into the vector. Expression of *p53* is directed by a highly efficient human cytomegalovirus immediate–early promoter–enhancer element.

4.2. Prior Studies on the System of Gene Transfer Including Risk Assessment Studies

The antineoplastic activity of various rAd/*p53* have been studied both in vitro and in vivo. In vitro studies using a number of different tumor cell lines have shown that Ad*p53* (i.e., wtp53–CMV–Ad) treatment produces a dose-dependent suppression of DNA synthesis, as measured by ^{3}H-thymidine incorporation ***(4)***. Ad*p53*-mediated suppression of tumor cell growth has been observed with a variety of *p53*-altered tumor cell lines, including those of colorectal, hepatocellular, non-small-cell lung, breast, and ovarian origin. Tumor cell lines, which are not *p53* altered, as well as nonmalignant cells in culture, exhibit little growth-suppression effects associated with Ad*p53*. In vivo studies have similarly shown tumor-suppressive effects associated with Ad*p53*-mediated gene therapy. The tumorigenicity in nude mice of human osteosarcoma (Saos-2) and human hepatocellular carcinoma (Hep3B) cells is suppressed if they are treated with Ad*p53 ex vivo* prior to implantation. Established tumor xenografts derived from human small cell and non-small-cell lung cancer cell lines have been treated with Ad*p53* given by local (peritumoral subcutaneous) administration resulting in suppression of tumor growth. In addition, a study of rAd/*p53* given locally to established tumor xenografts composed of H69 small-cell lung cancer cells has demonstrated increased survival in treated animals. In our laboratories, we have carried out work on three hepatocyte cell lines (one normal and two tumor) using the actual construct Ad*p53*. The results showed that wt-p53 expression led to killing of carcinoma cells, whereas it had no effect on the normal cells.

4.2.1. In Vitro Studies

Table 1 lists some of the published studies carried out to evaluate the potential of *p53* gene therapy of various human cancers using replication-deficient adenovirus as the vector. The methods applied included adenoviral infection of the various human cancer cell lines, Western blotting analysis, immunocytochemistry, checking for cell-cycle arrest, and analysis of cell viability. The

Table 1
In Vitro Studies

Tumor/Cancer	Transferred Nucleic Acid	Investigator	Results
Lung cancer cells	p53	Zhang et al. ***(5)***	Reduction in tumor size
Osteosarcoma cells	p53	Wang et al. ***(6)***	Apoptosis
Glioblastoma	p53	Gjerset et al. ***(7)***	Apoptosis
Colorectal cancer	Adp53	Spitz et al. ***(8)***	Apoptosis
Head and neck tumors	Adp53	Liu et al. ***(9)***	Cell growth arrest and apoptosis
Ovarian cancer	Adp53	Santoso et al. ***(10)***	Cell growth arrest and apoptosis
Ovarian cancer	Adp53	Mujoo et al. ***(11)***	Cell growth arrest and apoptosis
Glioblastoma	Adp53	Kock et al. ***(12)***	Cell growth arrest and apoptosis

results showed that at a multiplicity of infection (MOI) of 30, Ad*p53* infected >90% of cells derived from colon, brain, lung, breast, ovarian, and prostate cancer, but failed to infect leukemia or lymphomas cells. The infection resulted in nuclear accumulation of cyclin-dependent kinases inhibitor p21, cell-cycle arrest, and loss of viability.

These results are very promising and show that inhibition of cellular proliferation and cell killing effects of *p53* were dose dependent. These results agree with our in vitro cytotoxic assay findings carried out on liver cancer cell lines.

4.2.2. In Vivo Testing

Several in vivo evaluation studies involving the use of E1-deleted replication-deficient recombinant adenoviruses encoding wild-type human *p53* (under control of various promoters) showed that growth inhibition and/or regression of human tumors grown in animals can be safely and successfully achieved. The animal models included squamous cell carcinoma of the head and neck (SCCHN), colorectal, and lung cancers and others. The published results **(Table 2)** demonstrated that wt-p53

Table 2
In vivo studies (Animal Models)

Tumor/Cancer Nucleic Acid	Transferred Model	Animal	Investigator	Results
Colorectal cancer cells	p53	Mice	Harris et al. ***(13)***	In vitro growth inhibition and in vivo tumor growth suppression plus increased survival
Glioma	p53	Rats	Badie et al. ***(14)***	Inhibition of cell growth and induction of pheno typic changes
Squamous cell carcinoma of head and neck	p53	mice	Liu et al. ***(15)***	Apoptosis
"not tumor but intratracheal test" (please refer to publication title)	p53	Mice	Zhang et al. ***(16)***	Safety profile encouraging for clinical trials
Head and neck squamous carcinoma	p53	Mice	Clayman et al. ***(17)***	Prevented the establishment of subcutaneous tumors
Head and neck cancer cells	p53	Nude mice	Liu et al. ***(9)***	Growth arrest plus reduction in tumor size
Lung cancer cells	p53	Mice	Fujiwara et al. ***(18)***	Induction of massive apoptotic destruction of tumors
Head and neck cancer	p53	Mice	Clayman et al. ***(19)***	Significant inhibition of cell growth and significant reduction in tumor size
Small-cell lung carcinoma	p53	Mice	Wills et al. ***(4)***	Reduced tumor growth and increased survival

gene transfer and expression was sufficient to obtain the tumor response. Liu and colleagues ***(9)*** showed significant reduction in tumor volume in nude mice with established subcutaneous SCCHN nodules following peritumoral infiltration with the *p53* adenovirus. Their study included in vitro and in vivo evaluation of the effects of *the p53* adenovirus using cell culture, Northern blotting, Western blotting, immunohistochemistry, cell growth assay, and estimation of tumor volumes. In 1995, Liu et al. reported results of a similar study in which they characterized the mechanism of growth suppression by the exogenous *p53* gene as apoptosis ***(15)***. We performed an animal model for liver tumor and we were able to show that intratumoral injection of Adp53 resulted in tumor regression.

Harris and colleagues ***(13)*** studied several human carcinomas both in in vitro and in vivo after infection with the *p53* adenovirus. The studied cell lines contained wild-type, mutated, or no *p53* protein. One of their in vivo models used nude mice bearing subcutaneous human colon tumors that received peritumoral *p53*- adenovirus injection on a daily basis for 5 d. In this model, the average tumor volumes for the *p53* adenovirus-, blank adenovirus-, and buffer-treated groups were 600 mm^3, 1400 mm^3, and 3500 mm^3, respectively (i.e., *p53*-treated tumors were significantly smaller than buffer-treated controls [$p < 0.01$]). The analysis techniques used included cell culture, gene-expression detection, [3H]thymidine incorporation, and tumor volume estimation.

4.2.3. Clinical Protocols

Study 1: Has started in San Francisco. Sixteen patients with liver tumors were injected with Adp53 in the hepatic artery with doses up to 100 billion PFU. No toxicity was encountered.

Study 2: Twenty-four patients with advanced recurrent carcinoma of the head and neck were treated with direct injections of Adp53 with doses up to 10^{10} PFU. No adverse effects were encountered.

Study 3: Seventeen patients with non-small-cell lung cancer were treated with direct injection of Adp53 with doses up to 10^{12} PFU. No adverse effects were encountered.

Study 4: At the Cancer Gene Therapy meeting in London (1998), Dr. I. Ganly showed data of patients treated in Glasgow, with 10^{10} PFU attenuated adenovirus for head and neck tumors. There was no toxicity reported.

Study 5: Professor Tursz and colleagues report on six patients treated with up to 10^8 PFU Ad-RSV β-gal and found no toxicity. Subsequently, he gave an update of that study, in which he reported no toxicity in 10 patients with lung cancer receiving doses up to 10^9 PFU Ad-RSV β-gal.

Therefore, all five ongoing clinical trials **(Table 3)** using recombinant adenovirus reported no toxicity with three of these studies administering doses up to 10^{10} PFU. Three of these studies (Studies 1, 2, and 3) used Adp53. Studies

Table 3
Clinical Protocols Involving Use of Adenovirus Contructs

Study No.	Tumor/Cancer	Transferred Nucleic Acid	Protocol Title	Principal Investigator	Institute/Country
1	Colorectal Hepatocellular Liver metastases	Adp53	Gene therapy of primary and metastatic malignant tumors of the liver using Adp53 via hepatic infusion; a Phae I study	A. P. Venook and, R. S. Warren	University of California, San Francisco, CA
2	Head and neck squamous cell carcinoma	Adp53	Clinical protocol for modification of tumor suppressor gene expression in head and neck squamous cell carcinoma with an adenovirus vector expression wild-type p53	G.L. Clayman	UTMD Anderson Cancer Center, Houston, TX

3	Lung non-small-cell carcinoma	Adp53	Clinical protocol for modification of tumor suppressor gene expression and induction of apoptosis in non-small cell lung cancer with an adenovirus vector expressing wild-type p53 and cisplatin	J. A. Roth	UTMD Anderson Cancer Center, Houston, TX
4	Head and neck squamous cell carcinoma	ONYX 015	Clinical study with attenuated adenovirus that replicates in tumor cells lacking functional *p53*	Von Hoff/Kaye	USA/UK
5	Lung carcinoma	lacZ	Intratumoral transfer with recombinant vectors in lung cancer patients	T. Tursz	France

2 and 3 used the same vector and Study 1 used the intra-arterial route of administration in patients with primary and secondary liver tumors.

4.2.4. Risk Assessment

This procedure will only be used in patients over the age of 40 yr, in whom conventional therapeutic modalities have failed.

The risks to the patients are unforeseen effects of expression of *p53* within the tumor, the transmission of other biologically active products with the vector construct, and the clinical risks associated with percutaneous biopsy of the tumor. The risks seem to be negligible as 27 patients were treated in the United States with 10^{13} PFU (100 times more than the maximum proposed dose in this study) and had no serious side effects. The only abnormalities observed with 10^{13} PFU were low-grade fevers and transient elevation in liver function tests.

Participants in the trial will undergo a percutaneous, ultrasound-guided liver biopsy and the risk of this procedure is small. In a prospective series in 1000 patients, there were no deaths ***(20)*** and in a review of 68,276 biopsies, death occurred in 0.009% and serious complications, including hemorrhage, pneumothorax, and biliary peritonitis, in 0.3% ***(21)***. The risk to patients in this trial is expected to be less than these figures indicate because complications are more likely to occur in patients with cirrhosis of the liver and patients in this trial will not be cirrhotic.

In summary, the risk from overexpression of *p53* in the liver, of transmission of biological active substances with the vector construct, and of liver biopsy are expected to be very low.

4.3. Nature and Structure of the Vector

Defective adenovirus type 5 E1-deleted vector encoding human wild-type *p53* under the control of cytomegalovirus (CMV) major immediate–early (IE) promoter ***(22)***.

- cDNA of the *p53* obtained as a *Bam*HI fragment from pC53-SN3 plasmid (pCMV–Neo–Bam vector encoding wild-type *p53*).
- Fragment was cloned into the *Bam*HI site of the pMV10 vector ***(23)***.
- The *Hind*III fragment of pMV10–p53-wt was subcloned into the *Hind*III fragment of the pMV60 vector ***(23)***.
- The final pMV60–p53-wt plasmid was transfected into 293 cells.
- The wtp53-CMV-Ad recombinant was plaque purified and tested for production of transcriptionally active *p53* by infection of SW480-IAB3 (SW480 human colorectal cell line contransfected with a β-gal plasmid and pCMV–Neo–Bam, followed by selection with genetecin). This induced β-galactosidase activity in the infected cells.
- The wtp53–CMV–Ad was recombinant further purified by CsCl banding.

4.4. Overview of the Manufacturing Process of p53–CMV–Ad

4.4.1. Source of Seed Virus Stock

The original stock of virus was obtained from W. S. El-Deiry *(22)*.

4.4.2. Master Seed Virus Bank

A small Master Seed Virus Bank (MSVB) will be prepared from the seed material by two passages in 293 cells that complement the genes removed from the wtp53–CMV–Ad to render its replication incompetent. Master and Working Seed Cell Banks of 293 cells have been prepared by Magenta Microbiological Associates (now known as BioReliance Ltd.) under cGMP conditions and tested extensively for viral and other contaminants. The cell-culture process will use trypsin of approved origin that has been screened for porcine viruses by Magenta Microbiological Associates and culture media containing fetal calf serum (FCS) of Australian origin screened for bovine viruses by Magenta Microbiological Associates. The MSVB will be tested for sterility and mycoplasma and titrated for infectivity.

4.4.3. Manufacturing Process

The adenovirus clinical lot will be produced in cell culture using 293 cells, as earlier. The cells will be grown to near-confluency and infected at a multiplicity of infection of 7.5. Harvest will occur when a cytopathic effect has developed after a minimum 72-h infection. The virus will be released from the cell nuclei by three rounds of freeze–thaw followed by centrifugation to remove cellular debris.

Downstream processing will consist of two rounds of density gradient separation on cesium chloride, then a desalting step on a G-25 Sephadex column to remove salts including cesium. The adenovirus will be eluted with compound sodium lactate containing 10% extrapure (E.P.)-grade glycerol.

The bulk virus preparation will be tested for sterility and the ratio of defective to infective particles determined. It will then be diluted with compound sodium lactate containing 10% E.P.-grade glycerol and stored below –75°C in aliquots at the required volumes and titers suitable for the study.

4.4.4. Testing of the Producer Cell and Adenovirus Clinical Lot

4.4.4.1. 293 Cell Bank Testing

The following tests have been performed on the 293 master seed cell bank (MSCB):

- Sterility
- Mycoplasmas

- In vitro assay for detection of equine viral contaminants
- In vitro assay for detection of viral contaminants using MRC-5, Vero, HeLa, and RK13 detector lines
- In vivo assay for detection of viral contaminants using suckling and adult mice, guinea pigs, and embryonated eggs
- DNA Fingerprinting
- Transmission electron microscopic examination of at least 100 cells
- Reverse-transcriptase single-time-point assay with two templates
- Detection of bovine polyoma virus by PCR
- Detection of simian virus-40 (SV40) by PCR
- Extended in vitro assay with HeLa cells
- Detection of HIV-1 and HIV-2 by PCR
- Detection of HTLV-1 and HTLV-2 by PCR
- Detection of hepatitis B Virus by PCR
- Detection of hepatitis C Virus by PCR
- Detection of cytomegalovirus by PCR
- Detection of Epstein–Barr virus by PCR
- Detection of human parvoviruses by PCR
- Detection of papilloma viruses by PCR
- Detection of simian type D retroviruses SMRV, SRV-1, M-PMV (SRV-3) by DNA probe hybridization
- Detection of SIV-1 by PCR

The working seed cell bank (WSCB) was set up from the MSCB and tested for the following:

- Sterility
- Mycoplasmas
- DNA fingerprinting
- In vitro assay for detection of viral contaminants

4.4.4.2. Testing of the Vialed Clinical Lot

The following will be performed on the virus lot after final dilution and filling:

- Sterility
- Mycoplasma
- Identity by Southern blot analysis (Custom)
- In vitro assay for the detection of viral contaminants using MRC-5, Vero, and A549 cells
- LAL endotoxin test
- Test for abnormal toxicity/general safety in mice and guinea pigs (EP and USP)
- Detection of replication-competent adenovirus by cocultivation with A549 cells

4.5. Route

The wtp53-CMV-Ad will be administered in 50cc of normal (0.9%) saline and infused over 10–20 minutes in the surgically implanted pump catheter.

Dose escalation will occur until the maximum tolerated level or dose level 3 is achieved. Thereafter, a further 10 patients will receive the maximum tolerated level.

4.6. Study Design

This study seeks to determine the safety, biological efficacy, and effect of dose of wtp53–CMV–Ad in the loco-regional gene therapy of malignant tumors of the liver showing *p53* alteration. The study design consists of an open-label, nonrandomized, dose-escalation Phase I/II trial.

The wtp53–CMV–Ad will be administered by hepatic artery infusion to patients with malignant tumors of the liver. Study patients will be required to have evidence of *p53* alteration in tumor tissue. The study will include sampling on one occasion of normal and malignant tissue from the livers of patients following wtp53–CMV–Ad treatment. This will greatly facilitate assessments of clinical safety and biological efficacy, including efficiency and stability of gene transfer. Furthermore, sampling of treated tissues will require minimal additional morbidity for study patients.

5. Conduct of the Research

5.1. Recruitment/Selection of Subjects

5.1.1. Inclusion Criteria

Patients must fulfill all of the following criteria to be eligible for study admission:

Histologic diagnosis of colorectal carcinoma
For patients with colorectal carcinoma, documented metastatic tumor
The tumor(s) should have failed to respond to regional chemotherapy
Evidence of *p53* alteration in tumor tissue by immunohistochemistry
Patients with an infusion pump that was implanted for regional hepatic chemotherapy
At least 40 yr of age and less than 75 yr of age
Life expectancy of at least 3 mo
Adequate performance status (Karnofsky score $\geq$ 70%)
Required values for initial laboratory data (other tests are required; *see* **Subheading 5**):

WBC	$\geq$ 3000/L
Platelet count	$\geq$ 100000/L
Hct	$\geq$ 25% (may be transfused prior to enrollment)
PT	<20 s (control 15)
PTT	Within normal range
Creatinine	< 1.8 mg/dL or CrCl > 50 cm^3/min
Total bilirubin	< 2 mg/dL
AST, ALT	< 5 × upper limit of normal value

Preserved cardiopulmonary function:

SaO_2	$\geq$ 90% on room air
FEV_1	$\geq$ 70% or predicted value

Use of effective contraception during the trial and for 2 mo afterward.

5.1.2. Exclusion Criteria

Patients with any of the following will be excluded from study admission:

Pregnant
Uncontrolled serious bacterial, viral, fungal, or parasitic infection
HIV positive
Systemic corticosteroid therapy or other immunosuppressive therapy administered within the last 3 mo
Participation in another investigational therapy study within the last 3 mo
Any underlying medical condition that in the principal investigators' opinion will make the administration of wtp53–CMV–Ad hazardous or obscure the interpretation of adverse events
Karnofsky status less than 70%.

5.2. Prestudy Evaluation and Requirements

p53 status of tumor tissue in the liver (altered *p53* is an admission requirement). The following must be performed within 2 weeks prior to study admission (except where noted):

Complete history, physical examination, and toxicity evaluation, including:
- Performance status
- Height and weight, body surface area
- Baseline toxicity evaluation

Laboratory screening (*eligibility criteria):
- FBC with differential, platelet count*
- Serum electrolytes (sodium, potassium, chloride, bicarbonate), BUN, creatinine*, glucose, uric acid, albumin, total protein, calcium, phosphorus, and magnesium.
- AST,* ALT,* total bilirubin,* alkaline phosphatase, LDH, PT,* PTT*
- Urinalysis
- Electrocardiogram (12-lead)
- Chest x-ray (PA and lateral views)
- Abdomen and pelvis computed tomography or magnetic resonance imaging scan
- Tumor markers: carcino-embryonic antigen (CEA), CA 19.9

5.3.1. Informing and Seeking Consent from Possible Subjects of Research

Informed consent letter:

Hammersmith Hospital, Imperial College School of Medicine,
Informed Consent
Consent to Be a Research Subject

Gene Therapy of Tumors of the Liver Using wtp53-CMV-Ad Via Hepatic Artery Infusion: A Phase I/II Study

PURPOSE AND BACKGROUND

You have been invited to participate in this study because the cancer in your liver, unfortunately, cannot be removed surgically.

The Investigators wish to study a new way of treating cancer by gene therapy. In many types of cancer a particular gene, p53, is defective. It has been shown in the laboratory that putting back the correct form of this gene into cancer cells corrects their abnormal growth pattern. They now wish to see if this can be made to work in tumors such as yours, where the p53 gene is found to be abnormal. Before agreeing to take part in the study you are advised to contact your General Practitioner and organisations such as CancerBACUP for independent counselling. You may opt to join the study or you may continue with further chemotherapy or other medical support.

STUDY PROCEDURE

If you agree to participate the Investigators will perform various blood and urine tests, as well as taking a small sample of the tumor in your liver under biopsy. The treatment procedure itself will involve being admitted to the Hammersmith Hospital for an overnight stay. The investigators will inject the p53 gene via the pump that you will already have had inserted for previous chemotherapy. In order to ensure that the gene reaches the cells of the liver, it will have been 'packaged' in an adenovirus — a form of the common virus that causes colds and flu, but modified so that it does not lead to general infection of the body. The injection will be made in the pump that was implanted during the liver operation through which you received chemotherapy.

Two weeks before the treatment you will be checked in the out-patient clinic and blood samples will be taken on this occasion as well as during your stay in hospital. Further blood samples will be taken 1, 2, 3, 4 and 5 weeks after the treatment.

After the treatment you will be closely monitored in the hospital in a separate room before being discharged. One week later, you will have a biopsy to take samples of your tumor to see if the normal p53 protein is now being produced. You shall also be asked to continue coming to the Hospital for follow-up visits 2, 3, 4, 5 weeks and three monthly afterwards indefinitely (lifetime). The first tumor biopsy taken prior to this study was part of the routine management and was not being done for the trial. The second biopsy, however, is part of the study, and with any liver biopsy, there is a very small risk of bleeding. A CT scan will be performed 28 days after the treatment.

As this is a novel form of therapy, the risks associated with it are largely unknown. However, experiments suggest that the likelihood of serious side-effects are extremely small. Nevertheless, we would like you to use effective

contraception during the study and for two months thereafter. It is emphasized that at this stage of development the treatment is unlikely to have a beneficial effect on my tumor, although this is not impossible.

You are encouraged to ask any questions concerning the study.

REIMBURSEMENT

You will receive reimbursement of any taxi fares incurred as part of this study.

NEW FINDINGS

You and your family will be given any new information gained during the course of the study which might affect your willingness to continue your participation.

CONFIDENTIALITY

Participation in research may involve a loss of privacy. The results of all study tests will be discussed with you and your own doctor. Your medical records will be considered confidential and used only for medical and/or research purposes. Your identity will be kept confidential, and you will not be identified in any report or publication of this study or its results. Your NHS number and details of the trial in which you are participating will be provided to the Department of Health (DoH) so that your participation in this trial can be recorded on the National Health Service Central Register. This information will be used for purposes of long-term follow-up. You will not be contacted by DoH directly, but your GP may be asked to provide information on your health on occasion.

CONSENT

Participation in research is voluntary. I have the right to refuse to participate, or to withdraw from the study at any time. I may choose to do either one without affecting my relationship with my doctors and without affecting my future medical care. My participation may be ended at any time with or without my consent. If I wish to participate, I should sign below. I have been given copies of this document.

QUESTIONS AND ANSWERS

QUESTION Why have I been proposed to enter the gene therapy study?

ANSWER The tumor in your liver is resistant to surgery and chemotherapy. There is no conventional therapy currently available to you. Therefore we are proposing this new experimental therapeutic approach.

QUESTION What will happen to me if I do not take part in the study?

ANSWER You have the option for more chemotherapy or to continue with your existing medical support.

QUESTION How do I make up my mind?

ANSWER Take your time to think about it. Ask all the questions that come to mind. Discuss the study with your GP prior to consent. You can also ask for a second opinion from other specialists in other hospitals. If you are still unsure, then do not feel pressured to consent.

QUESTION What is the treatment schedule?

ANSWER You will be seen at first at the out-patient clinic, where you will have a clinical examination as well as blood tests. Two weeks later, you will be admitted to hospital. The drug will be injected into the reservoir that was implanted in your stomach during your last surgery. Numerous blood tests will be taken that day via an intravenous cannula inserted in your arm. You will stay in hospital overnight. The next day, further blood tests will be performed. If everything is fine, the intravenous cannula will be removed and you will be allowed to go home.

QUESTION What happens next?

ANSWER Seven days later you will be admitted to hospital for half a day. You will go to the X-Ray department and have a liver biopsy and more blood tests will be performed during that day.

QUESTION Is the liver biopsy risky?

ANSWER Yes, it is possible that you will bleed (Probability less than 1%) and death could occur (Probability less than one in a thousand).

QUESTION What happens after the biopsy?

ANSWER You will be seen every week for the following three weeks in the outpatient clinic and then every three months for a physical examination and blood test. On the twenty-eighth day after the treatment, you will also have a CT scan to determine the size of the tumor.

QUESTION Will I be followed up indefinitely?

ANSWER Yes, we wish to see you every three months thereafter in order to record your state of health.

QUESTION Will I benefit from the treatment?
ANSWER We have no evidence that the treatment will be effective.

QUESTION So why should I opt to take part in the study?
ANSWER You will help us understand cancer and help advance our knowledge in the treatment of this disease, so that this knowledge can then be used to benefit other cancer patients in the future.

5.3.2. Notification Letter to General Practitioner

Hammersmith Hospital, Imperial College School of Medicine
Letter to General Practitioner
Gene Therapy of Metastatic Tumors of the Liver Using wt*p53*-CMV-Ad
Via Hepatic Artery Infusion: A Phase I/II Study

Dear Doctor,

Please find enclosed an informed consent form that we gave to your patient Mr/Mrs _____ ,who is suffering from an irresectable liver tumor. We have offered this patient treatment with gene therapy which is a new experimental approach in this institution. We have asked the patient to come and see you in order to seek independent counselling.

We would like to draw your attention to the following:

1. It is a new treatment with complications mainly unknown.
2. We have no evidence that the treatment will be effective.
3. Complications could arise from the tumor biopsy such as bleeding.

We appreciate the time and effort you have given to help this patient with regard to advice and counselling. We are sending you the full protocol in order to provide you with appropriate information. Please get in touch with us if you need any further information.

5.4. Clinical and Technical Procedures

Treatment Plan

5.4.1. Route

The wtp53–CMV–Ad will be administered in 50cc of normal (0.9%) saline and infused over 10–20 min by percutaneous hepatic arterial catheter until the maximum tolerated level or dose level 3 are achieved. Thereafter, an additional 20 patients will receive the maximum tolerated level.

5.4.2. Schedule

Each patient will receive a single administration of wtp53–CMV–Ad via a hepatic arterial catheter.

5.4.3. Dose

5.4.3.1. Dosages will be calculated based on functional units of wtp53–CMV–Ad

5.4.3.2. Dose Levels

Level 1: 1.0×10^9 units
Level 2: 1.0×10^{10} units
Level 3: 3.0×10^{11} units

5.4.3.3.

The first three patients enrolled in the study will receive wtp53–CMV–Ad at dose level 1. If no dose-limiting toxicity (DLT) is observed after a period of at least 7 d of posttreatment monitoring (*see* **Subheading 5.4.5.1.**), the next three patients will receive Ad*p53* at dose level 2, and so on. If DLT is observed, the appropriate dose-escalation decision rule will be followed. This will be performed in parallel with patients with colorectal cancer.

5.4.3.4. Schema

Patients will be admitted to the Hammersmith Hospital on the day of treatment (day 1). wtp53–CMV–Ad will be infused via the surgically implanted pump.

After administration of wtp53–CMV–Ad serum samples will be obtained from a peripheral vein for pharmacokinetic analysis. Patients will remain in the ward for 48 h (d 1–2).

5.4.3.5. Flow Sheet

Day 14	Prestudy investigations and assessment
Day 1	Adenoviral injection
Day 2	Discharge
Day 7	Outpatient liver biopsy (6 h hospitalization)
Day 28	Outpatient assessment (1 h)

5.4.4. Study Evaluation

5.4.4.1. Immediate PostTreatment Monitoring

- Vital signs

Vital signs (body temperature, respiratory rate, heart rate, blood pressure) will be performed immediately prior to infusion, every 15 min during hour 1, and then at hours 2, 4, 8, 12, 16, 20, and 24 postinfusion. Vital signs will then be performed every 8 h until patient discharge.

- Shedding and excretion of wtp53–CMV–Ad
  Urine and stool will be collected from patients on d 1–2 and will be assayed for wtp53–CMV–Ad by a rapid on-site enzyme-linked immunosorbent assay (ELISA).

5.4.4.2. Study Evaluations, Days 1–2

These evaluations will be performed on d 1 prior to infusion and repeated on d 2, prior to discharge. This will include clinical evaluations as well as blood tests.

- Complete history, physical examination, and toxicity evaluation, including:
  - Performance status
  - Height and weight, body surface area
  - Toxicity evaluation
- Laboratory tests:
  - CBC with differential, platelet count
  - Serum electrolytes (sodium, potassium, chloride, bicarbonate), BUN, creatinine, glucose, uric acid, albumin, total protein, calcium, phosphorus, and magnesium
  - AST, ALT, total bilirubin, alkaline phosphatase, LDH
  - PT, PTT
  - Urinalysis
- Special studies
  - Pharmacokinetics (**Subheading 5.4.4.6.**)
  - Immune responses (**Subheading 5.4.4.7.**)
  - Study serum Bank (**Subheading 5.4.4.8.**)

5.4.4.3. Weekly Study Evaluations

These evaluations will be performed on d 7, 14, 21, and 28.

- Complete history, physical examination, and toxicity evaluation as above
- Laboratory tests as above
- Special studies
  - Pharmacokinetics
  - Immune responses
  - Study Serum Bank

5.4.4.4. Liver biopsies

Patients will undergo percutaneous Tru-cut liver biopsy of the liver tumor and normal liver as guided by ultrasound or CT. Liver biopsy will have been

performed prior to the study (as part of routine management) and 1 wk following the injection.

5.4.4.5. Poststudy and Follow-Up Evaluations

Patients will be considered to be actively on study from the time of study admission until the poststudy evaluations on d 28. Patients will be considered associated with the_study after the poststudy evaluations and will undergo regular follow-up evaluations for the duration of their lifetime.

The following evaluations will be performed on an outpatient basis on d 28 (poststudy evaluations), then every 3 mo for 1 yr, then yearly (follow-up evaluations), except as noted:

Complete history, physical examination, and toxicity evaluation.
Laboratory tests
Tumor markers:
Carcino-embryonic antigen (CEA) for patients with colorectal cancer (CRC).
Abdomen and pelvis CT or MRI scan to be performed on d 28 only. If tumor response is noted, abdominal scan will be repeated.
Special studies:

- Pharmacokinetics
- Immune responses
- Study serum bank

5.4.4.6. Pharmacokinetics

Serum samples will be obtained from the peripheral veins via an intravenous cannula for pharmacokinetic analysis.

Sampling of peripheral venous blood: Via an intravenous cannula inserted in a peripheral vein, 10cc of blood will be withdrawn, and then repeated at the additional times indicated. Timing of samples (time following the start of infusion): Preinfusion; 5, 15, and 30 min; 1, 6, 24, and 48 h; d 7, 14, 21, and 28.

5.4.4.7. Immune Responses

An important component of this study is to evaluate the immunogenicity of wtp53–CMV–Ad by measuring the serological response of treated patients to the adenoviral vector and to recombinant human wild-type *p53*.

Serologic responses to be assayed:

- Anti-adenovirus antibodies
- Anti-*p53* antibodies
- CD4 T-lymphocytes
- CD8 T-lymphocytes
- CD4 : CD8 T-lymphocytes subsets

Phlebotomy requirements: 10cc total of venous blood, concomitantly with the

samples for the Study Serum Bank (**Subheading 5.4.4.8.**).
Timing of samples: Preinfusion, Days 7, 14, 21, and 28, and as part of the Follow-Up Evaluations during the first year (**Subheading** 5.4.4.5.).

5.4.4.8. Study Serum Bank

Purpose: To preserve serum at specific time points for any further evaluations not listed in the study protocol.
Phlebotomy requirements: 10cc total of venous blood, concomitantly with the sample for the immune response assays (**Subheading 5.4.4.7.**).
Timing of samples: Preinfusion, d 7, 14, 21, and 28, and as part of the Follow-Up Evaluations during the first year (**Subheading 5.4.4.5.**).

5.4.4.9. Biological Efficacy Studies

One of the main objectives of this study is to assess the biological efficacy of wtp53–CMV–Ad, including the efficiency and stability of *p53* gene transfer. The molecular and cellular effects of wtp53–CMV–Ad treatment upon malignant tissue will be assayed.

Source of tissue: Malignant tissue from the Ad*p53*-treated liver will be obtained with Tru-cut biopsy.
Biological effects to be assayed or examined:
Distribution of Ad*p53* infection:
Cell types
Anatomic and microanatomic location.
p53 transduction efficiency and stability:
Proportion of tumor cells expressing transduced wild-type *p53*
Level of wild-type *p53* expression in transduced cells
Immunoblot with wild-type confirmation specific antibodies (McAb 1620)
Phenotypic effects: Cytologic changes associated with wtp53–CMV–Ad infection and/or wild-type *p53* transduction, as determined by histopathologic examination of normal and malignant tissue.
Inflammatory response: Evidence of inflammation as determined by histopathologic examination of normal and malignant tissue.

5.4.5. Potential Toxicity, Dose Modification, and Management

Toxicity will be assessed using the National Cancer Institute (NCI) criteria. Toxicity will be formally evaluation on d 1–2, 7, 14, 21, 28, then every 3 mo for 1 yr, then yearly.

5.4.6. Adverse Drug Reaction (ADR) Reports

Adverse Event Reporting Requirements

1. Patients will be instructed to report any adverse event to the investigators.
2. All adverse events occurring during participation in the study will be documented.
3. All adverse events will be reported to both Ethical Committee and GTAC, a descrip-

tion of the severity, duration, and outcome of the event, and the investigator's opinion regarding the relationship, if any, between the event and the study treatment.

5.4.7. Drug Preparation Formulation and Availability

The study drug, wtp53–CMV–Ad, will be prepared and provided at GMP grade by Magenta Microbiological Associates, UK (now known as BioReliance Ltd.).

5.4.8. Response Criteria

Tumor response to wtp53-CMV-Ad is among the primary objectives of this study. Observations of antitumor activity will be collected and analyzed.

Standard criteria will be formally employed to classify the antitumor responses observed in patients with measurable disease in the liver. Measurable disease will consist of bidimensionally measurable liver lesions with perpendicular diameters of $\geq$ 1 cm.

5.4.9. Removal from Study

Patients may withdraw or be removed from the study for any of the following reasons:

1. Patient's request to withdraw
2. Patient unwilling or unable to comply with study requirements
3. Clinical need for concomitant or ancillary therapy not permitted in the study
4. Any unacceptable treatment-related toxicity precluding further participation in the study.
5. Unrelated intercurrent illness that, in the judgment of the principal investigator, will affect assessments of clinical status to a significant degree

A patient removed from the study prior to any of the scheduled response evaluations will be considered inevaluable for response.

5.4.10. Concomitant Medications or Ancillary Therapy

1. Any specific antitumor treatment, including cytotoxic chemotherapy, hormonal therapy, biological therapy, radiation therapy, and oncologic surgery, must not be administered or performed while the patient is actively on study (from Study Admission to the time of Poststudy Evaluation; *see* **Subheading 5.4.4.5.**).
2. Patients must not receive any other investigational drugs while actively on study (from Study Admission to the time of Poststudy Evaluation).
3. Patients will receive full supportive care, including transfusions of blood and blood products, intravenous fluids, antibiotics, antiemetics, analgesics, and so forth, when clinically indicated.

5.4.11. Statistical Considerations: Sample Size

The sample size of three patients per dose level was selected to allow expeditious assessment of toxicity prior to further treatment.

5.4.12. Analysis

1. Pharmacokinetic analysis: The study will explore the relationships among pharmacokinetic parameters, toxicity, and biological efficacy.
2. Analysis of gene transfer efficiency: The study will explore the relationship between dose of wtp53–CMV–Ad and efficiency of transduction (gene transfer).
3. Analysis of clinical efficacy: Evaluation of clinical efficacy is one of the primary objectives of this study. Treatment effect will be estimated as the proportion of patients with objective responses (complete or partial response) following wtp53–CMV–Ad therapy. Chi-square tests and logistic regression will be used to analyze which variables are significant predictors of response.
4. Survival analysis: The Kaplan–Meier method will be used to estimate progression-free survival.

5.5. Follow-Up Arrangement

Follow-Up Evaluations

Patients will be considered to be actively on study from the time of Study Admission until the Poststudy Evaluations on d 28. Patients will be considered associated with the_study after the Poststudy Evaluations and will undergo regular follow-up evaluations for the duration of their lifetime. Consent will be obtained to enter their names into a register and for any future approaches that might be made to them in connection with monitoring. All patients will be followed and monitored for their lifetime.

5.6. Reporting Procedures

The investigators will report promptly any adverse side effects to the appropriate bodies. Progress reports will be filed with GTAC within 6 mo of the commencement of the study and at 6 mo intervals thereafter. In the event of a subject death, GTAC and MCA will be notified promptly. The findings at postmortem, including those of special studies together with a statement on cause of death, will be submitted as soon as possible.

6. Public Health Consideration

1. Precaution, testing, and measures to mitigate any risks to the public health. It is expected that the construct will not spread to other persons. Professor Tursz studied 10 patients treated with recombinant adenoviral vectors in lung cancer patients and found no cross-contamination to the medical and nursing staff. In the French study, there was no shedding of the virus beyond the third day. Therefore, we intend to keep the patients overnight in separate rooms with barrier nursing. We will analyze the urine and sputum of the medical and nursing staff during this period.
2. Exclusion of risks to offspring. In order to minimize the risk of cross-infection to offspring, only patients above the age of 40 will be included. It is unlikely that cancer patients above this age will remain reproductively active. Nevertheless, patients will

be warned of the risk and will be advised to take contraceptive measures.

3. Steps to Inform Staff. The protocol has been approved by our local Ethical Committee, which comprises medical and nursing staff as well as lay members of the public. The protocol has met the legal regulatory bodies (GTAC) and we will explain the proposed research to our medical and nursing staff that will be directly involved with the study. We will also inform the general practitioners of subjects of the study.

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Edited by

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