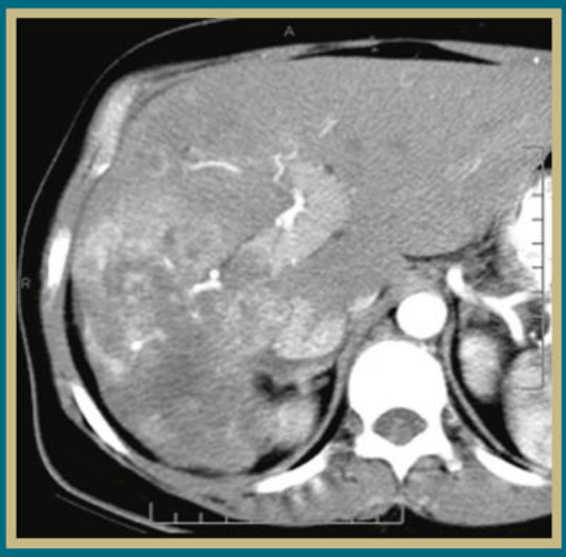
Hepatocellular Carcinoma

Diagnosis and Treatment

Second Edition

Edited by

Brian I. Carr



Humana Press

Hepatocellular Carcinoma

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To my daughters, Ophira and Feridey

Preface to 2nd Edition

You are not obliged to complete the task,
Nor are you free to stop trying.

—Talmud, Avot

Hepatocellular carcinoma (HCC) used to be regarded as a rare disease. The increasing numbers of chronic HCV carriers in the USA and subsequent increased incidence of HCC seen in most large medical centers mean that it is no longer an uncommon disease for gastroenterologists or oncologists to encounter, and its incidence and epidemiology are changing (new chapter). This has been enhanced by the appreciation that obesity (NASH or NAFL)-associated cirrhosis is also a cause of HCC, as are many metabolic syndromes (new chapter), in addition to carcinogens in the environment (new chapter), hepatitis B (new chapter), and hepatitis C (new chapter). Associated with this has been a clearer understanding of the many mechanisms involved in carcinogenesis of the liver (new chapter). During the period when liver resection and systemic chemotherapy were the only real therapeutic modalities available, the outcomes were generally dismal, especially since most patients presented with advanced-stage tumors. Several recent factors seem to have changed this. They include the more frequent use of aggressive surveillance by ultrasound and CT scanning in patients who have chronic hepatitis or cirrhosis from any cause and thus are known to be at risk for subsequent development of HCC in order to detect tumors at an earlier and thus more treatable stage. Advances in CT scanning, particularly the introduction of multi-head fast helical scans, mean that these vascular tumors can often be detected at an earlier stage or multiple lesions can now be appreciated, when only large single lesions were formally seen, so that unnecessary resections are not performed. Helical CTs have also largely replaced the more invasive CT arteriography. Furthermore, advances in MRI scanning (new chapter) have started to measure changes in tumor blood flow as a result of anti-angiogenic therapies (new chapter); so has dye-enhanced ultrasonography (new chapter). Liver transplantation has had a profound effect on the therapeutic landscape. There have always been two hopes for this modality, namely to eliminate cirrhosis as a limiting factor for surgical resection and also to extend the ability of the surgeon to remove ever-larger tumors confined to the liver. The organ shortage for patients with HCC who could be transplanted has been alleviated in part by two

new factors. They are the MELD criteria, which give extra points to patients with small tumors, and the introduction of live donor transplants (new chapter), which obviate the need for long waits for a cadaveric donor. Regional chemotherapy and hepatic artery chemoembolization have been around for a long time and have been practiced mainly in the Far East and in Europe. There has not been a consensus on which drug or drug combinations are best or even whether embolization is important, and if so, what type and size of embolizing particle might be optimal. While there is still no consensus on these matters, it has recently become clear from two randomized controlled clinical trials that hepatic artery chemoembolization for unresectable, non-metastatic HCC seems to bestow a survival advantage compared with no treatment. The high recurrence rates after resection have led numerous investigators to evaluate pre-resection and post-resection chemotherapy in the hope of decreasing recurrence rates. Only recently have clinical trials begun to provide evidence of enhanced survival for multimodality therapy involving resection with added chemotherapy or ^{131}I lipiodol. The introduction of ^{90}Y microspheres (Theraspheres) appears to offer the promise of relatively non-toxic tumoricidal internal radiotherapy to the liver and appears to be a major therapeutic addition to our treatment choices, and its role alone or in combination with other therapies is just beginning to be explored. The advent of multiple clinical trials for new agents that inhibit either the cell cycle or angiogenesis or both (new chapter) has diminished enthusiasm for chemotherapy, since these agents appear to be less toxic and may enhance survival, even for advanced disease. Some of these agents are taken orally, which makes them even more attractive. In addition, we are beginning to enter the phase of genomics (new chapter) and proteomics (new chapter) as applied to many tumor types, including HCC. This raises the possibility of being able to categorize patients into prognostic subsets, prior to any therapy. We are just at the beginning of the age of cell cycle modulating factors including hormones, growth factors, and growth factor receptor antagonists and agents that specifically alter defined aspects of the cell cycle. Since the mechanisms of many of these agents are known, we are entering the era of personalized medicine and the rational selection of suitable treatment drugs for an individual patient. For all these reasons, it seemed reasonable to us to produce a book that presents much of current therapy and current thinking on HCC. This is an exciting time to be in the field of HCC basic science as well as clinical management, since so many changes are simultaneously occurring at multiple levels of our understanding and management of the disease, and suddenly there are many new choices of therapy to offer our patients. All the original chapters have also been updated and enhanced.

Philadelphia, PA Brian I. Carr
March, 2009

Preface to 1st Edition

You are not obliged to complete the task,
nor are you free to desist from trying.

—Talmud, Avot

Hepatocellular carcinoma (HCC) used to be regarded as a rare disease. The increasing numbers of chronic hepatitis C virus carriers in the United States and subsequent increased incidence of HCC seen in most large medical centers means that it is no longer an uncommon disease for most gastroenterologists or oncologists to encounter.

During the times when liver resection or systemic chemotherapy were the only real therapeutic modalities available, the outcomes were generally dismal, especially because most patients presented with advanced-stage tumors. Several recent factors seem to have changed this. They include the more frequent use of aggressive surveillance by ultrasound and computed tomography (CT) scanning in patients who have chronic hepatitis or cirrhosis from any cause (and thus are known to be at risk for subsequent development of HCC) to detect tumors at an earlier and therefore more treatable stage. Advances in CT scanning, particularly the introduction of multihead fast helical scans, mean that this vascular tumor can often be detected at an earlier stage, or multiple lesions can be diagnosed when only large single lesions were formerly seen, so that unnecessary resections are not performed.

Liver transplantation has had a profound effect on the therapeutic landscape. There have always been two hopes for this modality: namely, to eliminate cirrhosis as a limiting factor for surgical resection and also to extend the ability of the surgeon to remove ever-larger tumors confined to the liver. Regional chemotherapy and hepatic artery chemoembolization have been around for a long time and have been practiced mainly in the Far East and Europe.

There has not been a consensus for which drug or drug combination is best or whether embolization is important and, if so, what type and size of particle are optimal. Although there is still no consensus on these matters, it has recently become clear from two randomized controlled clinical trials that hepatic artery chemoembolization for unresectable nonmetastatic HCC seems to bestow a survival advantage compared to no treatment. The high recurrence rates after resection have led numerous

investigators to evaluate preresection and postresection chemotherapy in the hope of decreasing recurrence rates. Only recently have clinical trials begun to provide evidence of enhanced survival for multimodality therapy involving resection and either chemotherapy or 131I-lipiodol. The introduction of 90Yttrium microspheres, which appear to offer the promise of relatively nontoxic tumoricidal therapy to the liver, appears to be a major therapeutic addition to our treatment choices, and its role alone or in combination with other therapies is just beginning to be explored.

In addition, we are beginning to enter the phase in which proteomics is applied to many tumor types, including HCC. This raises the possibility of being able to categorize patients into prognostic subsets, prior to any therapy. We are also just at the beginning of the age of cell cycle modulating factors including hormones, growth factors, and growth factor receptor antagonists and agents that specifically alter defined aspects of the cell cycle.

For these reasons, it seemed reasonable to produce a book that represents much of the current therapy and thinking on HCC. Admittedly, there is a bias toward expressing the experience of one center, the Liver Cancer Center at the University of Pittsburgh Starzl Transplant Institute, in which over 250 new cases of HCC have been seen each year for the last 15 years. This is an exciting time to be in the field of HCC basic science as well as clinical management because so many changes are simultaneously occurring at multiple levels of our understanding and management of the disease.

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1 Epidemiology of Hepatocellular Carcinoma

Donna L. White, PhD, MPH, Amir Firozi, MD, and Hashem B. El-Serag, MD, MPH

CONTENTS

ABSTRACT

Hepatocellular carcinoma (HCC) affects more than half a million individuals per year worldwide. It is a largely preventable disease. Most cases are related to hepatitis B virus infection in sub-Saharan Africa and Eastern Asia (except Japan). Hepatitis C virus has emerged as an important cause of HCC particularly in North America and some parts of Europe, where a recent sharp increase in HCC has been reported. There is growing evidence of an association between obesity and diabetes and increased risk of HCC; however, the causal link is still unclear. The striking geographic and racial variations in the occurrence of HCC are partly explained by the distribution of HBV and HCV infections. Additional established risk factors for HCC include older age, male sex, heavy alcohol intake, aflatoxin exposure, iron overload related to hemochromatosis, and possibly tobacco smoking. The

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role of diet except for alcohol drinking and aflatoxin contamination in the etiology of HCC in human populations is largely unknown. Host genetic factors are being examined but definitive data are lacking. Most of these risk factors operate by promoting the development of cirrhosis which is present in most HCC cases. The annual risk of HCC in cirrhosis ranges between 1 and 7%. This review discusses in detail the epidemiology of HCC from a global perspective.

Key Words: Hepatitis C; hepatitis B; cirrhosis; incidence; prevalence; risk; genetic association; coffee; insulin resistance; liver cancer; epidemiology; determinants; risk factors

1. GLOBAL INCIDENCE OF HEPATOCELLULAR CARCINOMA

1.1. Overview

Primary liver cancer is the fifth most common cancer worldwide and the third most common cause of cancer mortality *(1)*. Globally, over 560,000 people develop liver cancer each year and an almost equal number, 550,000, die of it. Liver cancer burden, however, is not evenly distributed throughout the world (Fig. 1). Most HCC cases (>80%) occur in either sub-Saharan Africa or in Eastern Asia. China alone accounts for more than 50% of the world's cases (age-standardized incidence rate (ASR) male: 35.2/100,000; female: 13.3/100,000). Other high-rate (>20/100,000) areas include Senegal (male: 28.47/100,000; female: 12.2/100,000), The Gambia (male: 39.67/100,000; female: 14.6/100,000), and South Korea (male: 48.8/100,000; female: 11.6/100,000).

North and South America, Northern Europe, and Oceania are low-rate (< 5.0/100,000) areas for liver cancer among most populations. Typical incidence rates in these areas are those of the United States (male: 4.21/100,000; female: 1.74/100,000), Canada (male: 3.2/100,000; female: 1.1/100,000), Colombia (male: 2.2/100,000; female: 2.0/100,000), the United Kingdom (male: 2.2/100,000; female: 1.1/100,000), and Australia (male: 3.6/100,000; female: 1.0/100,000). Southern European countries, typified by rates in Spain (male: 7.5/100,000; female: 2.4/100,000), Italy (male: 13.5/100,000; female: 4.6/100,000), and Greece (male: 12.1/100,000; female: 4.6/100,000), are of medium rate (5.0–20.0/100,000) *(2)*.

HCC accounts for between 85 and 90% of primary liver cancer. One noteworthy exception is the Khon Kaen region of Thailand, which has one of the world's highest rates of liver cancer ($ASR_{1993–1997}$ male: 88.0/100,000; female: 35.4/100,000) *(3)*. However, due to endemic infestation with liver

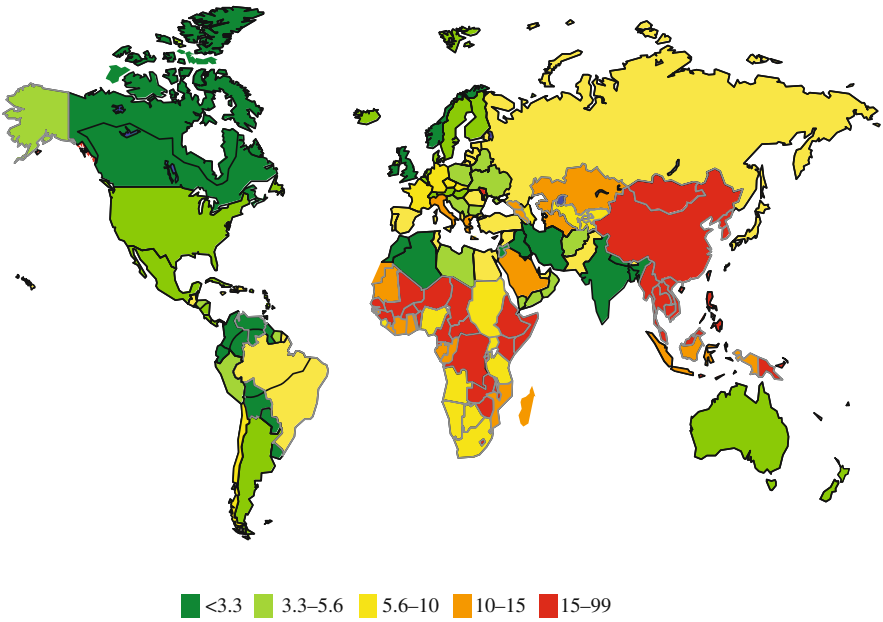

Fig. 1. Regional variations in the incidence rates of hepatocellular carcinoma categorized by age-adjusted incidence rates.

flukes, the major type of liver cancer in this region is intrahepatic cholangiocarcinoma rather than HCC *(4)*.

Encouraging trends in liver cancer incidence have been seen in some of these high-rate areas *(5)*. Between 1978–1982 and 1993–1997, decreases in incidence were reported among Chinese populations in Hong Kong, Shanghai, and Singapore *(3)*. In addition to these areas, Japan also began to experience declines in incidence rates among males for the first time between 1993 and 1997 (Fig. 2).

Many high-rate Asian countries now vaccinate all newborns against HBV and the effect on HCC rates has already become apparent. In Taiwan, where national newborn vaccination began in 1984, HCC rates among children aged 6–14 years declined significantly from 0.70/100,000 in 1981–1986 to 0.36/100,000 in 1990–1994 *(6)*. It is too soon yet for HBV vaccination to have had an effect on adult rates, but other public health measures may have contributed to declines in HCC incidence in high-risk areas of China. A Chinese government program started in the late 1980s to shift the staple diet of the Jiangsu Province from corn to rice may have limited exposure to known hepatocarcinogen aflatoxin B1 (AFB1) in this area *(7)*. Similarly, another Chinese public health campaign initiated in the early 1970s to encourage drinking of well water rather than pond- or ditch water may have decreased

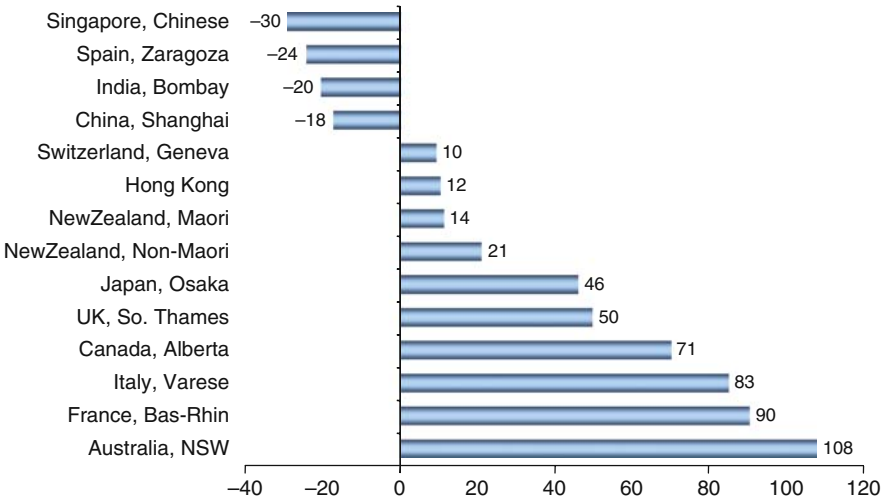

Fig. 2. Recent changes in the incidence of HCC. The incidence of HCC has been declining in some "high-incidence" areas, such as China and Hong Kong. On the other hand, HCC incidence in several "low and intermediate incidence" areas has been increasing. Modified from McGlynn et al. *(5)*.

consumption of microcystins, cyanobacteria-produced compounds demonstrated to be hepatocarcinogenic in experimental animals.

In contrast, registries in a number of low-rate areas reported increases in HCC incidence between 1978–1982 and 1993–1997. Included among these registries are those in the United States, the United Kingdom, and Australia. Reasons for both the decreased incidence in high-rate areas and the increased incidence in low-rate areas are not yet clear, suggesting that each area will be an important case study. It has been widely hypothesized, however, that increased incidence in low-rate areas may be related to greater prevalence of HCV infection within these areas.

1.2. Race/Ethnicity

HCC incidence rates also vary greatly among different populations living in the same region. For example, ethnic Indian, Chinese, and Malay populations of Singapore had age-adjusted rates ranging from 21.21/100,000 among Chinese males to 7.86/100,000 among Indian males between 1993 and 1997 *(3)*. The comparable rates for females were 5.13/100,000 among ethnic Chinese and 1.77/100,000 among ethnic Indians. Another example is the United States where, at all ages and among both genders, HCC rates are two times higher in Asians than in African-Americans, which are themselves two times higher than those in whites. The reason(s) for this interethnic

variability likely include differences in prevalence and acquisition time of major risk factors for liver disease and HCC.

1.3. Gender

In almost all populations, males have higher liver cancer rates than females, with male:female ratios usually averaging between 2:1 and 4:1. At present, the largest discrepancies in rates (>4:1) are found in medium-risk European populations. Typical among these ratios are those reported from Geneva, Switzerland (4.1:1) and Varese, Italy (5.1:1). Among 10 French registries listed in volume VIII of *Cancer in Five Continents*, nine report male:female ratios >5:1. In contrast, typical ratios currently seen in high-risk populations are those of Qidong, China (3.2:1); Osaka, Japan (3.7:1); The Gambia (2.8:1); and Harare, Zimbabwe (2.4:1). Registries in Central and South America report some of the lowest sex ratios for liver cancer. Typical ratios in these regions are reported by Colombia (1.2:1) and Costa Rica (1.6:1).

The reasons for higher rates of liver cancer in males may relate to gender-specific differences in exposure to risk factors. Men are more likely to be infected with HBV and HCV, consume alcohol, smoke cigarettes, and have increased iron stores. Higher levels of androgenic hormones, body mass index, and increased genetic susceptibility may also adversely affect male risk.

1.4. Age

The global age distribution of HCC varies by region, incidence rate, gender and, possibly, by etiology *(3)*. In almost all areas, female rates peak in the age group 5 years older than the peak age group for males. In low-risk populations (e.g., the United States, Canada, the United Kingdom), the highest age-specific rates occur among persons aged 75 and older. A similar pattern is seen among most high-risk Asian populations (e.g., Hong Kong, Shanghai). In contrast, male rates in high-risk African populations (e.g., The Gambia, Mali) tend to peak between ages 60 and 65 before declining; while female rates peak between 65 and 70 before declining. These variable age-specific patterns are likely related to differences in the dominant hepatitis virus in the population, the age at viral infection and the existence of other risk factors. Notably, while most HCV carriers became infected as adults, most HBV carriers became infected at very young ages.

Exceptions to these age patterns occur in Qidong, China, where liver cancer rates are among the world's highest. Age-specific incidence rates among males rise until age 45 and then plateau, while among females, rates rise

until age 60 and then plateau. The explanation for these younger peak ages is unclear, but may be due to existence of other hepatocarcinogenic exposures.

1.5. Distribution of Risk Factors

Major risk factors for HCC vary by region. In most high-risk areas, the dominant risk factor is chronic HBV infection. In Asia, HBV infection is largely acquired by maternal–child transmission, while sibling-to-sibling transmission at young ages is more common in Africa. Consumption of aflatoxin B_1-contaminated foodstuffs is the other major HCC risk factor in most high-rate areas.

Unlike the rest of Asia, the dominant hepatitis virus in Japan is hepatitis C (HCV). HCV began to circulate in Japan shortly after World War II *(8)*. Consequently, HCC rates began to sharply increase in the mid-1970s with an anticipated peak in HCV-related HCC rates projected around 2015, though recent data suggests the peak might have already been reached.

In low-rate HCC areas, increasing numbers of persons living with cirrhosis is the likely explanation for rising HCC incidence. This has resulted from a combination of factors including rising incidence of cirrhosis due to HCV and, to a lesser extent, HBV infection, as well as a general improvement in survival among cirrhosis patients. It has been estimated that HCV began to infect large numbers of young adults in North America and South and Central Europe in the 1960s and the 1970s as a result of intravenous drug use *(9)*. The virus then moved into national blood supplies and circulated until a screening test was developed in 1990, after which time rates of new infection dropped dramatically. Currently, it is estimated that HCV-related HCC in low-rate countries will peak around 2010.

1.6. HCC in the United States

Age-adjusted HCC incidence rates increased more than 2-fold between 1985 and 2002 *(10)* (Fig. 3). Average annual, age-adjusted rate of HCC verified by histology or cytology increased from 1.3 per 100,000 during 1978–1980 to 3.3 per 100,000 during 1999–2001 *(11)*. The increase in HCC started in the mid-1980s with greatest proportional increases occurring during the late 1990s. The largest proportional increases occurred among whites (Hispanics and non-Hispanics), while the lowest proportional increases occurred among Asians. The mean age at diagnosis is approximately 65 years, 74% of cases occur in men, and the racial distribution is 48% white, 15% Hispanic, 13% African-American, and 24% other race/ethnicity (predominantly Asian). During recent years as incidence rates increased, the age distribution of HCC patients has shifted toward relatively younger ages, with greatest proportional increases between ages 45 and 60.

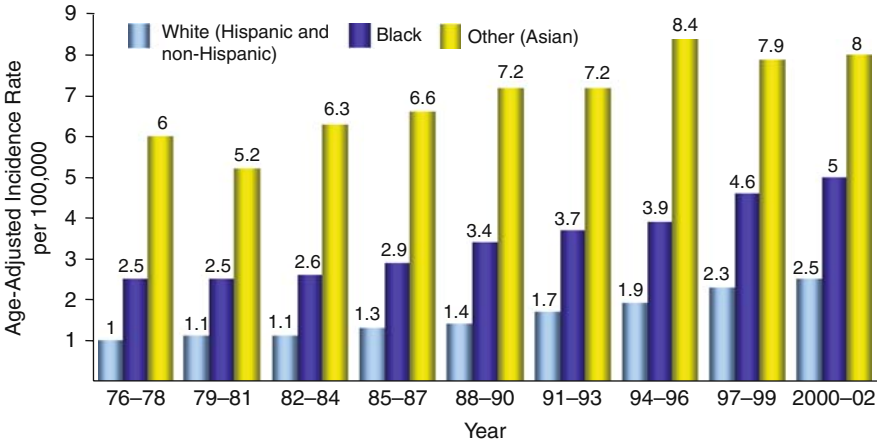

Fig. 3. Average yearly, age-adjusted incidence rates for HCC in the United States shown for 3-year intervals between 1975 and 2002. Whites include approximately 25% Hispanic while other race is predominantly Asian (88%).

Four published studies examined secular changes in HCC risk factors in the United States *(12–15)*. Two studies were from large, single referral centers where viral risk factor ascertainment was based on serology findings, while the other two were from national databases in which risk factors were ascertained from ICD-9 codes in billing or discharge records. In all four studies, the greatest proportional increases occurred in HCV-related HCC, while HBV-related HCC had the lowest and most stable rates. Overall, between 15 and 50% of HCC patients in the United States have no established risk factors.

2. RISK FACTORS OF HEPATOCELLULAR CARCINOMA

HCC is unique in that it largely occurs within an established background of chronic liver disease and cirrhosis (~70–90% of all detected HCC cases) (Fig. 4). Major causes of cirrhosis in patients with HCC include hepatitis B, hepatitis C, alcoholic liver disease, and possibly, non-alcoholic steatohepatitis.

2.1. Hepatitis B Virus

Globally, HBV is the most frequent underlying cause of HCC with an estimated 300 million persons with chronic infection worldwide. Case–control studies have demonstrated that chronic HBV carriers have a 5- to 15-fold increased risk of HCC compared to the general population.

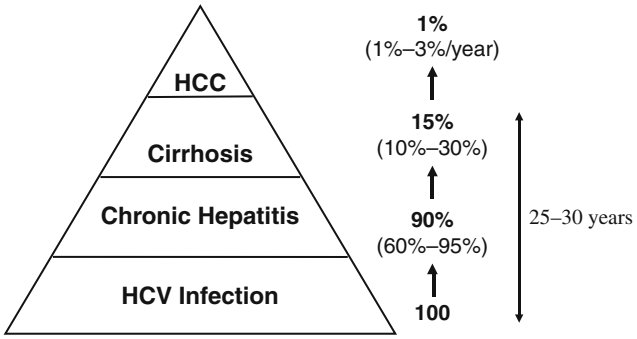

Fig. 4. Estimated progression rates to cirrhosis and hepatocellular carcinoma in hepatitis C infection.

The great majority, between 70 and 90%, of HBV-related HCC develops in a background of cirrhosis. HBV DNA is found in the host genome of both infected and malignant hepatic cells. HBV may, therefore, initiate malignant transformation through a direct carcinogenic mechanism by increasing likelihood of viral DNA insertion in or near proto-oncogenes or tumor-suppressor genes. However, despite initial excitement accompanying this discovery, subsequent research has failed to show a unifying mechanism by which integration of HBV DNA leads to HCC.

The increased HCC risk associated with HBV infection particularly applies to areas where HBV is endemic. In these areas, it is usually transmitted from mother to newborn (vertical transmission) and up to 90% of infected persons follow a chronic course. This pattern is different in areas with low-HCC incidence rates where HBV is acquired in adulthood through sexual and parenteral routes (horizontal transmission) with >90% of acute infections resolving spontaneously. The annual HCC incidence in chronic HBV carriers in Asia ranges between 0.4 and 0.6%. This figure is lower in Alaskan natives (0.26%/year) and lowest in Caucasian HBV carriers *(16)*.

Several other factors have been reported to increase HCC risk among HBV carriers including male gender; older age (or longer duration of infection); Asian or African race; cirrhosis; family history of HCC; exposure to aflatoxin, alcohol, or tobacco; or coinfection with HCV or HDV. HCC risk is also increased in patients with higher levels of HBV replication, as indicated by presence of HBeAg and high HBV DNA levels. In addition, it has been suggested in Asian studies that genotype C is associated with more severe liver disease than genotype B *(17)*.

In the natural history of chronic HBV infection, spontaneous or treatment-induced development of antibodies against HBsAg and HBeAg leads to improved clinical outcomes. A meta-analysis of 12 studies with 1,187 patients who received interferon and 665 untreated patients followed for

5 years found lower HCC incidence in treated 1.9% (95% CI 0.8–3.0%) than untreated patients 3.2% (95% CI 1.8–4.5%). However, this difference was not statistically significant *(18)*.

Using sensitive amplification assays, many studies have demonstrated that HBV DNA persists as "occult HBV infection" for decades among persons with serological recovery (HBsAg negative) from acute infection. Occult HBV is associated with anti-HBc and/or anti-HBs *(19)*. However, in a significant proportion of individuals, neither anti-HBc nor anti-HBs can be detected. A single multinational investigation found prevalence of occult HBV in liver tissue to be 11% in Italy, 5–9% in Hong Kong, and 0% in the United Kingdom. Supporting an association with occult HBV, a high proportion of individuals with HCV infection who develop HCC have demonstrable HBV DNA and proteins in their neoplastic and adjacent non-neoplastic liver tissue. However, although some studies have linked development of HCC in individuals with chronic HCV infection to occult HBV, others have not found an association.

2.2. Hepatitis C Virus

Chronic HCV infection is a major risk factor for development of HCC. Markers of HCV infection are found in a variable proportion of HCC cases; for example, 44–66% in Italy, *(20, 21)* 27–58% in France, 60–75% in Spain, and 80–90% in Japan *(8)*. A higher but undefined proportion of HCC patients might have had HCV detected by PCR testing of liver tissue and/or serum, even if antibody to HCV (anti-HCV) was non-detectable. In a meta-analysis of 21 case–control studies in which second-generation enzyme immunoassay tests for anti-HCV were used, HCC risk was increased 17-fold in HCV-infected patients compared with HCV-negative controls (95% CI 14–22) *(22)*.

The likelihood of development of HCC among HCV-infected persons is difficult to determine due to the paucity of adequate long-term cohort studies; however, the best estimate is from 1 to 3% after 30 years (Fig. 5). HCV increases HCC risk by promoting fibrosis and eventually cirrhosis. Once HCV-related cirrhosis is established, HCC develops at an annual rate of 1–4%; though rates up to 7% have been reported in Japan. Rates of cirrhosis 25–30 years post-infection range between 15 and 35% *(23)*. The highest incidence rates were observed in HCV-contaminated blood or blood products recipients (14 and 1 per 1000 person-years for cirrhosis and HCC, respectively) and in hemophiliacs (5 and 0.7 per 1000 person-years). The lowest rates have been reported in women who received a one-time contaminated anti-D immune globulin treatment (1 and 0 per 1000 person-years, respectively).

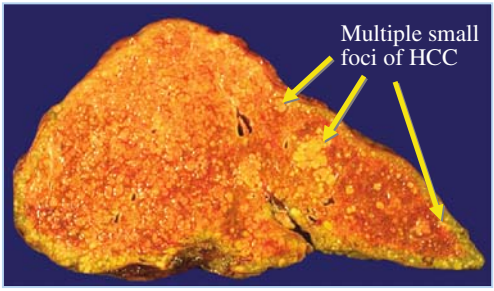

Fig. 5. Cirrhosis and hepatocellular carcinoma. Explanted liver showing features of cirrhosis and multiple small foci of HCC throughout the liver in a miliary pattern (*arrows*).

In HCV-infected patients, factors related to host and environment/lifestyle appear to be more important than viral factors in determining progression to cirrhosis. These factors include older age, older age at the time of acquisition of infection, male gender, heavy alcohol intake (>50g/day), diabetes, obesity, and coinfection with HIV or HBV *(24)*. There is no strong evidence that HCV viral factors like genotype, viral load, or quasispecies are important in determining the risk of progression to cirrhosis or HCC.

Successful antiviral therapy in patients with HCV-related cirrhosis may reduce future risk of HCC, but the evidence is weak. There is only one prospective, randomized, controlled trial that examined the effects of antiviral therapy on HCC, a Japanese trial in which 100 patients were randomized to receive either 6 million units of interferon alfa thrice weekly for 3–6 months or were followed without treatment *(25)*. After a 2- to 7-year follow-up period, HCC was significantly reduced in the treated (4%) compared to the non-treated control group (38%), a 93% reduction in adjusted risk. However, much of this risk reduction was a result of the unusually high HCC rate among these controls. Other studies, mostly retrospective and non-randomized, suggested moderately decreased HCC risk among HCV-infected patients treated with interferon *(26–37)*.

In general, reported preventive effects of interferon therapy were less marked in European compared to Japanese studies. However, the lack of randomization in most of these studies may exaggerate treatment benefits as it is likely that healthier patients tend to get treated more frequently than those with advanced liver disease (who are known to be more likely to develop HCC). In addition to a role in primary prevention of HCC among HCV-infected patients, a few Japanese reports suggest interferon may also be effective for secondary prevention in individuals who have previously undergone resection for HCC.

2.3. Alcohol

Heavy alcohol intake, defined as ingestion of >50–70 g/day for prolonged periods, is a well-established HCC risk factor. It is unclear whether risk of HCC is significantly altered in those with low or moderate alcohol intake. Although heavy intake is strongly associated with development of cirrhosis, there is little evidence of a direct carcinogenic effect of alcohol otherwise.

There is also evidence for a synergistic effect of heavy alcohol ingestion with HCV or HBV, with these factors presumably operating together to increase HCC risk by more actively promoting cirrhosis. For example, Donato et al. *(22)* reported that among alcohol drinkers, HCC risk increased in a linear fashion with daily intake >60 g. However, with concomitant presence of HCV infection, there was an additional 2-fold increase in HCC risk over that observed with alcohol usage alone (i.e., a positive synergistic effect).

2.4. Aflatoxin

Aflatoxin B_1 (AFB_1) is a mycotoxin produced by the *Aspergillus* fungus. This fungus grows readily on foodstuffs like corn and peanuts stored in warm, damp conditions. Animal experiments demonstrated that AFB_1 is a powerful hepatocarcinogen leading the International Agency for Research on Cancer (IARC) to classify it as carcinogenic *(30)*.

Once ingested, AFB_1 is metabolized to an active intermediate, AFB_1-*exo*-8,9-epoxide, which can bind to DNA and cause damage, including producing a characteristic mutation in the p53 tumor-suppressor gene (p53 249^{ser}) *(29)*. This mutation has been observed in 30–60% of HCC tumors in aflatoxin endemic areas *(27, 36)*.

Strong evidence that AFB_1 is a risk factor for HCC has been supplied by person-specific epidemiological studies performed in the last 15 years. These studies were permitted by development of assays for aflatoxin metabolites in urine, AFB_1-albumin adducts in serum, and detection of a signature aflatoxin DNA mutation in tissues.

Interaction between AFB_1 exposure and chronic HBV infection was revealed in short-term prospective studies in Shanghai, China. Urinary excretion of aflatoxin metabolites increased HCC risk 4-fold while HBV infection increased risk 7-fold. However, individuals who both excreted AFB_1 metabolites and were HBV carriers had a dramatic 60-fold increased risk of HCC *(38)*.

In most areas where AFB_1 exposure is a problem, chronic HBV infection is also highly prevalent. Though HBV vaccination is these areas should be the major preventive tactic, persons already chronically infected will not

benefit from vaccination. However, HBV carriers could benefit by eliminating AFB_1 exposure. Efforts to accomplish this goal in China *(7)* and Africa *(36)* have been launched.

2.5. Non-alcoholic Fatty Liver Disease (NAFLD) and Non-alcoholic Steatohepatitis (NASH)

Studies in the United States evaluating risk factors for chronic liver disease or HCC have failed to identify HCV, HBV, or heavy alcohol intake in a large proportion of patients (30–40%). It has been suggested that many cryptogenic cirrhosis and HCC cases, in fact, represent more severe forms of non-alcoholic fatty liver disease (NAFLD), namely non-alcoholic steatohepatitis (NASH). Potential risk factors such as diabetes, obesity, and possibly HCV are likely to increase HCC risk at least partly by promoting NAFLD and NASH.

One difficulty in epidemiological studies attempting to elucidate the association between NASH and risk of HCC in humans, however, is that once either cirrhosis or HCC is established, it is difficult to identify pathological features of NASH. Several clinic-based case–control studies have, in fact, indicated that HCC patients with cryptogenic cirrhosis tend to have clinical and demographic features suggestive of NASH (predominance of women, diabetes, obesity) than age- and sex-matched HCC patients of well-defined viral or alcoholic etiology *(2–4)*. For example, Regimbeau et al. examined 210 patients who underwent resection for HCC of whom 18 (8.6%) had no identifiable cause for chronic liver disease and found higher prevalence of obesity (50% vs. 17% vs. 14%) and diabetes (56% vs. 17% vs. 11%) compared to patients with alcoholic and viral hepatitis, respectively *(39)*. Evidence of progression from NAFLD to HCC from prospective studies is scant. There are case reports *(5, 6)* and a small case series describing development of HCC several years following NASH diagnosis *(40)*. In a community-based retrospective cohort study, 420 patients diagnosed with NAFLD in Olmsted County, MN, were followed for a mean duration of 7.6 years. In that study, liver disease was the third leading cause of death (as compared with the 13th leading cause of death in the general Minnesota population) occurring in seven (1.7%) subjects. Twenty-one (5%) patients were diagnosed with cirrhosis of whom two developed HCC *(5, 6, 8)*.

2.6. Diabetes

Diabetes, particularly type II diabetes, has been proposed to be a risk factor for both chronic liver disease and HCC through development of NAFLD and NASH. It is known to contribute significantly to hepatic steatosis *(9, 10)*

with development of increased levels of steatosis associated with more severe necroinflammatory activity *(11, 12)* and fibrosis *(16–18)*. Fibrosis progression rates have also appeared to be higher when marked steatosis was present *(19)*, with some studies suggesting that the increase in steatosis itself may be an indicator of fibrosis progression *(13)*. Additionally, liver disease occurs more frequently in those with more severe metabolic disturbances, with insulin resistance itself demonstrated to increase as liver disease progresses *(20)*.

Several case–control studies from the United States, Greece, Italy, Taiwan, and Japan examined the association between diabetes, mostly type II, and HCC. At least eight studies found a significant positive association between diabetes and HCC, two found a positive association that did not quite reach significance, and one found a significant negative association. A potential bias in cross-sectional and case–control studies, however, is difficulty in discerning temporal relationships between exposures (diabetes) and outcomes (HCC). This problem is relevant in evaluating HCC risk factors because 10–20% of patients with cirrhosis have overt diabetes and a larger percentage have impaired glucose tolerance. Thus, diabetes may also be the result of cirrhosis.

Cohort studies, which are intrinsically better suited to discern temporal relationships between exposure and disease, have also been conducted. All compared HCC incidence in cohorts of diabetic patients to either the expected incidence given HCC rates in the underlying population or the observed HCC incidence among a defined cohort without diabetes *(41)*. Three studies conducted among younger or smaller cohorts found either no or low number of HCC cases. At least four other cohort studies examined large number of patients for relatively long time periods, with three studies finding significantly increased risk of HCC with diabetes (risk ratios ranging between 2 and 3) *(21–23)*. We recently conducted a study of HCC incidence in a large cohort of VA patients ($n = 173{,}643$ with and $n = 650{,}620$ without diabetes). The findings of this study indicate HCC incidence doubled among patients with diabetes and was higher among those with longer duration of follow-up *(41)* (Fig. 7).

While most studies have been conducted in low-HCC rate areas, diabetes has also been found to be a significant risk factor in areas of high HCC incidence like Japan. Further, although other underlying risk factors like HCV may confound the association between diabetes and HCC, they do not seem to fully explain it. Taken together, available data suggest that diabetes is a moderately strong risk factor for HCC *(42)*. However, additional research is needed to more fully examine how any excess risk conveyed by diabetes is mediated by such potentially confounding factors as duration and treatment of diabetes, family history of diabetes, and current and historical levels of obesity and physical activity.

2.7. Obesity

Obesity, especially abdominal obesity, is strongly correlated with insulin resistance and type II diabetes, a state of clinically diagnosable advanced insulin resistance that has itself been associated with HCC risk. Some evidence in support of a direct contribution of obesity-mediated metabolic errors in hepatocarcinogenesis comes from experimental research in a genetically obese ob/ob knockout mouse model of NAFLD that demonstrated hepatic hyperplasia even at very early stages of disease and without evidence of cirrhosis *(25)*.

The effect of obesity on HCC risk has been examined in several cohort studies. In a large prospective cohort study of more than 900,000 individuals from around the United States followed for a 16-year period, liver cancer mortality rates were five times greater among men with the greatest baseline BMI (35–40) compared to those with normal BMI *(43)* (Fig. 6). In the same study, the risk of liver cancer was not as elevated in women with a relative risk of 1.68 (0.93–3.05). Two other population-based cohort studies from Sweden and Denmark found excess HCC risk (elevated relative risk of 2- to 3-fold) in obese men and women compared to those with normal BMI *(44, 45)*. The effects of obesity on HCC risk may vary according to the presence of other underlying risk factors for HCC; however, the data are consistent. In a large prospective cohort study in Taiwan, obesity (BMI 30+) conveyed excess risk of HCC even after controlling for other metabolic risk factors including presence of diabetes mellitus *(26)*. The greatest increase in risk with obesity was observed in the context of HCV infection (HR = 4.10, 95% CI 1.38–12.4). While a 2.4-fold excess risk that approached significance was also observed among persons who were negative for both HBV and HCV infection, obesity conveyed only a very modest and non-significant 1.4-fold excess risk among persons with HBV

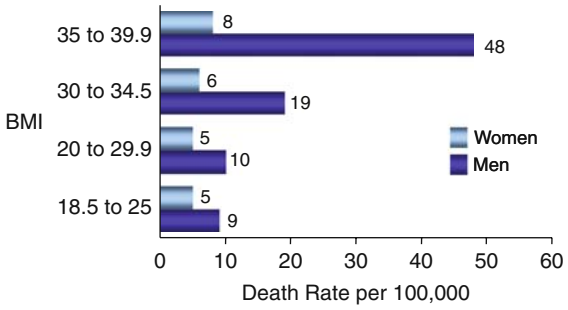

Fig. 6. Obesity and liver cancer. In both men and women, a higher body mass index (BMI) is significantly associated with higher rates of death due to cancer of the liver. Modified from Calle et al. *(43)*.

infection. There was, however, evidence of very strong synergism between obesity and diabetes which, when both conditions occurred together, conveyed a 100-fold excess HCC risk with obesity in the context of either HBV or HCV infection. In a retrospective study of over 19,000 registry-listed individuals in the United states with cirrhosis who received a liver transplant, the effect of obesity on HCC risk also varied according to disease etiology *(46)*. Specifically, obesity conveyed strong and significant excess risk of HCC even after controlling for presence of diabetes among transplant recipients with cryptogenic or alcoholic cirrhosis (OR = 11.1, 95% CI 1.5–87.4 and OR = 3.2, 95% CI 1.5–6.6, respectively). However, obesity was not an independent predictor of HCC risk among those with other disease etiologies including HCV or HBV infection, biliary cirrhosis, or autoimmume hepatitis.

Several case–control studies have also evaluated the association between BMI and risk of HCC. In a study in Japan conducted in chronically HCV-infected patients, the incidence of HCC was significantly increased among those with a higher BMI. Further, there was also evidence of a dose-dependent relationship with a significant 1.8-fold excess HCC risk in HCV+ cases who were overweight (BMI 25–<30) that increased to a 3.1-fold excess in those who were obese (BMI 30+) in comparison to lean HCV+ cases *(33)*. Another case–control study conducted in a regional medical center in the United States compared the prevalence of obesity among 70 HCC cases to that observed among 140 age- and gender-matched controls ($n = 70$ with cirrhosis and $n = 70$ without liver disease) *(47)*. HCC cases were significantly more likely to be obese than either patients with cirrhosis or normal controls (OR = 4.3, 95% CI 2.1–8.4 and OR = 47.8, 95% CI 9.6–74.5). Further, there was evidence of significant synergism or particularly increased risk of HCC among those with obesity (BMI 30+) who also had more than 100 drinks and smoked more than 100 cigarettes during their lifetime (OR = 7.4, 95% CI 2.1–14.6). Although this study did not include adjustment for presence of diabetes, the overall prevalence of diabetes was similar among the HCC case, cirrhotic case, and normal control groups.

Taken together the data suggest that obesity conveys excess risk of HCC beyond that conveyed by diabetes. However, the actual magnitude of risk and the specific subgroups of chronic liver disease patients in whom its presence may be most salient in promoting HCC risk varied across studies. Future research with evaluation of additional factors that may influence obesity-mediated risk of HCC including timing and duration of obesity as well as family history of obesity and diabetes may be helpful in identifying subgroups of obese chronic liver disease patients who may particularly benefit from enhanced surveillance and therapeutic interventions.

In conclusion, many developing countries are in the midst of a burgeoning obesity epidemic. This is particularly apparent in the United States where

a recent national study found that 30% of all adults (60+ million) are obese (i.e., BMI 30+) *(48)* and 16% of all children (9+ million) are overweight (i.e., BMI-for-age $\geq$ 95th percentile per CDC Growth Charts) *(49)*. Although the exact magnitude and mechanisms of obesity-mediated HCC risk are currently unknown, even small increases in obesity-mediated risk could translate into a large number of HCC cases.

2.8. Tobacco

The relationship between cigarette smoking and HCC has been examined in more than 50 studies in both low- and high-rate areas. In almost all countries, both positive association and lack of association findings have been reported. Among studies reporting positive associations, several found effects were limited to population subgroups defined by HBV status, HCV status, genetic polymorphism, or other exposure. Taken together, available evidence suggests that any effect of smoking on HCC is likely to be weak and limited to a subset of the general population. However, because two studies conducted exclusively among women reported positive associations, it has been suggested that attributable risk among women may be higher than that in men *(50, 51)*.

2.9. Oral Contraceptives

The association between oral contraceptives use and HCC risk was examined in at least 12 case–control studies ($n = 740$ cases and $n = 5{,}223$ controls) *(52)*. The pooled estimator was OR = 1.43 (95% CI 0.90–2.26, $p = 0.13$). Six studies showed a significant 2- to 20-fold increase in HCC risk with longer durations (>5 years) of oral contraceptives use. Whether newer, low-dose oral contraceptives convey similar potential risks is currently unknown.

2.10. Diet

The role of diet, except for alcohol drinking and aflatoxin contamination, in the etiology of HCC in human populations is largely unknown. Dietary anti-oxidants including selenium as well as retinoic acid and beta-carotene have been shown to inhibit hepatocarcinogenesis in animals. However, epidemiologic data are fairly limited and in some places conflicting. In a cohort study of men in Taiwan, higher baseline levels of serum retinol were associated with a decreased risk of developing HCC in HBV carriers. In the same cohort, a lower vegetable intake was significantly associated with an increased risk of HCC; however, this effect was limited to individuals who

were both chronic hepatitis B carriers and cigarette smokers *(53)*. In a subsequent report from the same cohort, low baseline serum levels of selenium were also predictive of increased HCC risk *(54)*. In another large cohort study in Japan, the only foods whose consumption conveyed significantly decreased risk of HCC in subjects without a known history of liver disease was fish, while the only food that conveyed decreased risk in subjects with a history of liver disease was coffee. Another study among Japanese atomic bomb survivors reported an approximately 50% reduction in HCC risk among those with high consumption of miso soup and tofu, both rich in the antioxidant isoflavones, after adjusting for HBV and HCV viral infections *(55)*.

Several studies performed in Southern Europe, predominantly in Italy, have also evaluated various dietary factors as potential risk or protective factors for HCC. A favorable effect of high intake of specific foods including milk and yogurt, white meats, eggs, and fruits and of selected macronutrients including beta-carotene was reported by a multicenter hospital-based case–control study in Italy *(56)*. A similar inverse association between vegetable and fruit consumption and risk of HCC was also demonstrated in another much smaller case–control study in Italy. On the other hand, a smaller case–control conducted in Athens, Greece, did not support an association between vegetable intake or any other specific foods or nutrients with risk of HCC, with the possible exception of milk/dairy products which conveyed a modestly decreased risk that closely approached significance *(57)*.

Coffee Drinking: One of the most extensively studied dietary factors in relation to HCC risk in human populations is coffee drinking. Several epidemiological studies have previously reported coffee drinking reduces risk of elevated liver enzymes and of cirrhosis, while animal studies suggest that coffee reduces liver carcinogenesis. Further, coffee drinking has also been associated with reduced insulin levels as well as reduced risk of type II diabetes, itself considered to be a risk factor for HCC *(42)*. At least nine epidemiological studies conducted in Japan and Southern Europe specifically evaluated the relationship between coffee consumption and HCC risk. Coffee drinking was associated with reduced HCC risk in at least five case–control studies (25–75% risk reduction with two to four cups of coffee per day as compared to none) *(42, 58–61)*. Three cohort studies have also reported on the association between coffee intake and subsequent risk of HCC *(62–64)*. Of those, two studies showed significant reduction in HCC risk with coffee intake of one or more cups of coffee, and of those, with one further showing a dose–response relationship (20% reduction with one to two cups and 75% reduction with five or more cups) *(63)*. Although the third publication reporting on two cohorts also showed reduced HCC risk with coffee drinking, its findings were only of borderline significance *(62)*. One potential limitation of most of these studies is that they used general

population controls, which may not be the most appropriate comparator group given their low background risk for HCC as well as for chronic liver disease. However, the inverse association between coffee consumption and HCC persisted in the studies that presented results stratified by liver disease *(42, 60, 62, 65)* or used a second control group of patients with liver disease *(61)*. Taken together these data suggest a modest reduction of HCC risk with coffee drinking. However, the specific components of coffee and the exact mechanisms by which they act to reduce HCC risk are not well established.

Overall, there is increasing evidence suggesting that dietary factors may play a role in promoting hepatocarcinogenesis. However, there are important gaps in the epidemiologic literature that limit broad generalizations about the role specific dietary factors may play in HCC risk both within and across populations. First, studies published to date have used a variety of instruments to assess dietary intake. Even with use of validated instruments, there is well-known difficulty in reliably measuring dietary intake which is further complicated by differences in the relevant time period for which dietary intake was assessed. Second, many studies did not adequately account for factors that may confound the relationship between actual and biologically effective intake of specific micro- and macronutrients including obesity and physical activity. Finally, most studies have been performed in Southeast Asian and Southern European populations. It is unclear whether results obtained solely within those populations would generalize to other populations including those of Northern Europe and North America where there are differences in the underlying risk factors for HCC, dietary patterns, and potentially confounding factors like obesity and diabetes.

3. GENETIC EPIDEMIOLOGY OF HCC

Although a very small minority of HCC cases are associated with familial disorders of Mendelian inheritance like hereditary hemachromatosis, alpha-1-antitrypsin deficiency, or porphyrias, epidemiological research has convincingly demonstrated that the great majority of adult-onset HCC cases are sporadic (i.e., have no similarly affected first-degree relative) and that many have at least one established non-genetic risk factor like habitual alcohol abuse or chronic infection with hepatitis B or C viruses. However, most people with these known environmental risk factors for HCC never develop cirrhosis or HCC, while a sizable minority of HCC cases develop among individuals without any known environmental risk factors.

Genetic variation has long been suspected to influence the variable risk for HCC observed both within and across populations. Familial clusters of disease have been observed in HCC in the context of HBV infection *(66, 67)*

as well as among those without established risk factors *(68)*. As most HCC cases are sporadic or have no similarly affected first-degree relative, interest in the role commonly inherited genetic variants may play as potential risk factors for HCC has grown.

Currently, far fewer genetic epidemiological studies have been reported for HCC than for other more common cancers in developed countries, like lung, prostate, or breast cancers. Most studies in area of HCC have been case–control studies conducted in populations with high HCC rate (Asian, African) or medium rate (European). Typically, they have examined only a limited number of polymorphisms within a few genes selected because of (1) their role in the key liver function of detoxification including Phase I and Phase II enzymes like cytochrome P-450s (CYPs), *N*-acetyltransferases (NATs), and glutathione *S*-transferases (GSTs); (2) their role in biological pathways potentially relevant in chronic liver disease and carcinogenesis including inflammatory response (e.g., interleukins (ILs) 1β, IRN) and DNA repair (e.g., XRCC1); or (3) their role in mitigating or exacerbating the effects of exposure to specific etiologic risk factors for HCC like alcohol or aflatoxin (e.g., ADH3, ALDH2, EPHX1).

Results from the genetic epidemiology studies evaluating varied polymorphisms, including CYPs *(69–71)*, NATs *(72, 73)*, GSTs *(74, 75)*, ILs *(76, 77)*, and ALDH2 *(78, 79)*, as risk factors for HCC have largely been equivocal, with findings of a positive association, association only within a limited subset of the population, or no or negative association all reported. The lack of reproducibility is a phenomenon widely reported in the broader field of genetic epidemiology. It has been widely attributed to inadequate sample sizes to reliably detect the likely small effects of common genetic variants on risk, particularly within a background of strong environmental risk factors and with likely polygenic influences on development of disease *(80, 81)*. Furthermore, virtually all of these studies have lacked power to detect interactions; it is estimated that several thousand cases and controls are required to adequately assess the effects of gene–gene or gene–environment interactions. Other contributing factors include population stratification or population-based differences in the relative distribution of alleles (e.g., among different racial groups), use of non-representative control groups, variable genetic penetrance, and potential differences in relevant genes based on underlying etiology of liver disease (e.g., alcohol or hepatitis related).

Given genetic epidemiology studies are often highly underpowered, meta-analysis has been recognized as an important tool to more precisely define the effect of individual polymorphisms on relative risk of disease and to identify potentially important sources of between-study heterogeneity *(82, 83)*. We recently completed a meta-analysis evaluating the effect of the two most frequently evaluated polymorphisms for HCC risk to date,

the dual deletion of GST polymorphisms *GSTM1* ($n = 14$ studies) and *GSTT1* ($n = 13$ studies) *(84)*. Individual studies for both polymorphisms reported variable findings and therefore the observed heterogeneity necessitated use of a random-effects model. Pooled estimators suggested a possible small excess risk with either *GSTM1* or *GSTT1* null genotypes, though findings approached significance only for *GSTT1* ($OR_{GSTM1} = 1.16$, 95% CI 0.89–1.53, $OR_{GSTT1} = 1.19$, 95% CI 0.99–1.44). Exploratory meta-regressions suggested source of the controls was a possible source of observed between-study heterogeneity, with greater risk among hospital-based controls for both polymorphisms. Year of publication was an additional source of between-study heterogeneity for *GSTM1* only. Although overall pooled estimators for *GSTM1* and *GSTT1* suggest a possible small excess of HCC with the null genotype, additional studies with larger samples and conducted in other populations are needed to further clarify the role of both polymorphisms in the etiology of HCC and to investigate gene–environment interaction.

The epidemiologic literature evaluating selected SNPs as HCC risk factors is currently limited to case–control studies of only small to modest size. Therefore, a particularly noteworthy recent advance in the field of genetic epidemiology is the development of large-scale cohorts or DNA "biobank" cohorts that will be prospectively followed for development of disease (e.g., biobanks in the United Kingdom ($n = 500{,}000$) and Mexico ($n = 200{,}000$)) *(85)*. These large-scale genetic cohort studies offer many important advantages over traditional case–control studies including the ability to validly discern temporal relationships between exposure and disease and the availability of an appropriate control group. However, in spite of their impressive sample size, given the rarity of HCC and the considerable latency until

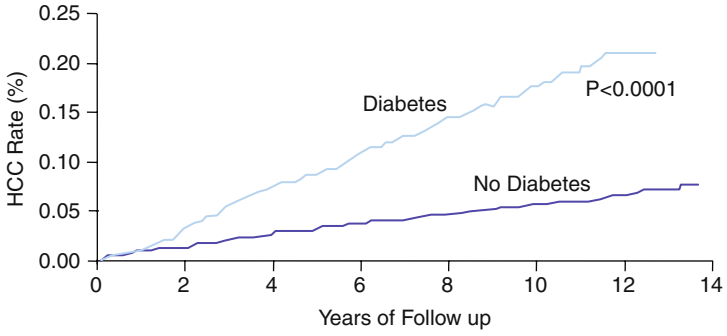

Fig. 7. The cumulative incidence of HCC among veteran patients hospitalized between 1985 and 1990. The study examined 173,463 patients with diabetes and 650,620 without diabetes. No patient had acute or chronic liver disease recorded before, during, or within 1 year of their index hospitalization.

disease onset, they are unlikely to generate enough HCC cases to fully replace genetic case–control and disease-based registry studies.

Overall, as in other areas of genetic epidemiology, results of studies in HCC have fallen short of early expectations that they would rapidly and unequivocally result in identification of genetic variants conveying substantial excess risk of disease and thereby establish the groundwork for effective genetic screening for primary prevention. However, recent identification of genetic risk factors for some chronic diseases such as Alzheimer's disease and breast cancer, development of multidisciplinary efforts to address the considerable complexity in identifying genetic risk factors, and the increasing accessibility to technology to concomitantly evaluate many thousands of SNPs across the genome (i.e., genome-wide association studies) have contributed to a "cautious optimism" *(85)* that genetic epidemiology will ultimately provide important information on etiopathogenesis of many chronic diseases. Efforts within the field of gastroenterology to promote use of best practice in genetic epidemiologic research may facilitate identification of genetic risk factors for particular diseases of interest including HCC *(86)*.

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2 Environmental Carcinogens and Risk for Human Liver Cancer

John D. Groopman, Kimberly Brodovicz, and Thomas W. Kensler

CONTENTS

ABSTRACT

Collectively liver cancer, including hepatocellular carcinoma (HCC) and cholangiocarcinoma, accounts for 5.7% of all reported cancer cases and is the sixth most common cancer diagnosed worldwide. The incidence of liver cancer varies enormously globally and unfortunately the burden of

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this nearly always fatal disease is much higher in the economically less developed countries of Asia and sub-Saharan Africa. This chapter will review the significant data that link exposures to specific environmental carcinogens and the development of HCC in many parts of the world. These epidemiologic studies have been made possible by devising biomarkers reflective of exposure and risk. The translation of these basic science findings to an understanding of the etiology of HCC has also provided guidance for the development of preventive interventions in high-risk populations. Thus, the consistency of the experimental animal and human data points to the important role that environmental exposures play in gender differences in HCC risk.

Key Words: Hepatocellular carcinoma (HCC); Cholangiocarcinoma; aflatoxin B_1 (AFB_1); environmental exposures; biomarkers; hepatitis B surface antigen (HBsAg); hepatitis B virus (HBV); hepatitis C virus (HCV)

1. INTRODUCTION

Collectively liver cancer, including hepatocellular carcinoma (HCC) and cholangiocarcinoma, accounts for 5.7% of all reported cancer cases and is the sixth most common cancer diagnosed worldwide *(1)*. The incidence of liver cancer varies enormously globally and unfortunately the burden of this nearly always fatal disease is much higher in the economically less developed countries of Asia and sub-Saharan Africa (Fig. 1) *(2)*. HCC is also the most rapidly rising solid tumor in the United States and is overrepresented in minority communities, including African-Americans, Hispanic/Latino-Americans, and Asian-Americans *(3)*. Overall, there are more than 650,000 new cases each year and over 200,000 deaths annually in the People's Republic of China (PRC) alone *(4, 5)*. In contrast with most common cancers in the economically developed world where over 90% of cases are diagnosed after the age of 45, in high-risk regions for liver cancer onset begins to occur in both men and women by 20 years of age and peaks between 40 and 49 years of age in men and between 50 and 59 years of age in women *(1, 6, 7)*. This earlier onset of HCC might be attributable to exposures that are both substantial and persistent across the life span. Gender differences in liver cancer incidence have also been described and the worldwide annual age-standardized incidence rate among men is 15.8 per 100,000 and 5.8 per 100,000 among women *(8)*. These epidemiologic findings are also similar to experimental animal data for one potent liver carcinogen linked to human HCC, aflatoxin, and male rats have been found to have an earlier onset of cancer compared to female animals *(9)*. Thus, the consistency of the experi-

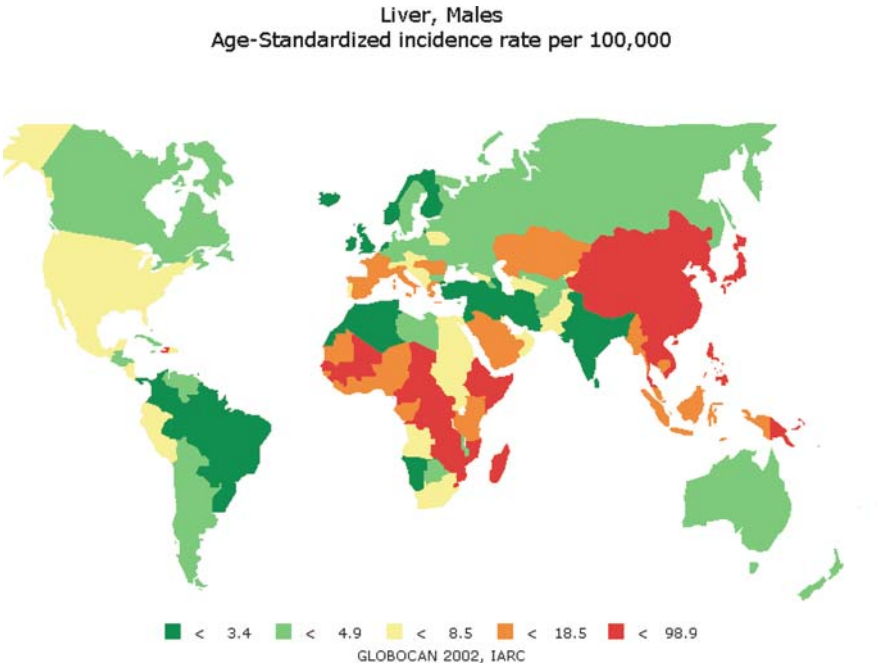

Fig. 1. Age-standardized incidence of liver cancer in men worldwide *(8)*.

mental animal and human data points to the important role that environmental exposures play in gender differences in HCC risk.

This chapter will review the significant data that link exposures to specific environmental carcinogens and the development of HCC in many parts of the world. These epidemiologic studies have been made possible by devising biomarkers reflective of exposure and risk. The translation of these basic science findings to an understanding of the etiology of HCC has also provided guidance for the development of preventive interventions in high-risk populations. We will review a number of these major investigations to provide an overview of this very active field of research.

2. MOLECULAR BIOMARKERS FOR ENVIRONMENTAL CARCINOGENS

Molecular biomarkers are typically used as indicators of exposure, effect, or susceptibility for both individuals and communities. A biomarker of exposure refers to measurement of the specific compound of interest, its metabolite(s), or its specific interactive products in a body compartment or fluid, which indicates the presence and magnitude of current and past exposure. A biomarker of effect indicates the presence and magnitude of a biological

response from exposure to an environmental agent. Such a biomarker may be an endogenous component, a measure of the functional capacity of the system, or an altered state recognized as impairment or disease. A biomarker of susceptibility is an indicator or a metric of an inherent or acquired ability of an individual to respond to the challenge of exposure to a specific toxicant. Such a biomarker may be the unusual presence or absence of an endogenous component, or an abnormal functional response to an administered challenge *(10)*. Measures of these biomarkers through molecular epidemiology studies have great utility in addressing the relationships between exposure to environmental agents and development of clinical diseases, and in identifying those individuals at high risk for the disease *(11, 12)*. These data also help to inform the risk assessment process, where the effectiveness of regulations can be tested against biological measurements of exposure and effect.

The validation of any biomarker–effect link requires parallel experimental and human studies *(13)*. Following the development of a hypothesis of an exposure disease linkage, there is the need to devise the analytical methodology necessary to measure these biological markers in human and experimental samples. Conceptually, an appropriate animal model is often used to determine the associative or causal role of the biomarker in the disease or effect pathway, and to establish relations between dose and response. The putative biomarker can be validated in pilot human studies, where sensitivity, specificity, accuracy, and reliability parameters can be established. Data obtained in these studies can then be extended to assess intra- or interindividual variability, background levels, relationship of the biomarker to external dose or to disease status, as well as feasibility for use in larger population-based studies. To fully interpret the information that the biomarker provides, prospective epidemiological studies may be necessary to demonstrate the role that the biomarker plays in the overall pathogenesis of the disease or effect. Finally, these biomarkers can be translated as intermediate endpoints in interventions in both experimental models and high-risk human populations to optimize agent selection, dose, and schedule and other parameter influencing efficacy.

3. ENVIRONMENTAL ETIOLOGY OF HCC

As described above, HCC is among the leading causes of cancer death in most parts of the economically developing world. The unequal distribution of this disease is depicted by the map in Fig. 1 based upon the IARC cancer database *(8)*. Since the level of HCC is also coincident with regions where aflatoxin exposure is high, many efforts starting over 40 years ago examined this possible association. These initial studies were hindered by the lack of adequate data on aflatoxin intake, excretion, and metabolism in people, the underlying susceptibility factors such as diet and viral exposure,

as well as by the incomplete statistics on worldwide cancer morbidity and mortality. Despite these deficiencies, early studies did provide data illustrating that increasing HCC rates corresponded to increasing levels of dietary aflatoxin exposure *(14)*. The commodities most often found to be contaminated by aflatoxin were common human food staples including: peanuts, cottonseed, corn, and rice *(15)*. The requirements for aflatoxin production are relatively non-specific since molds can produce these toxins on almost any foodstuff and the final levels in the grain product can vary from microgram to tens of milligrams *(16)*. Indeed, in a recent case of aflatoxin-related deaths in rural villages in Kenya, daily exposures were estimated to be over 50 mg *(17)*. Because contamination of foodstuffs is so heterogeneous, the measurement of human exposure to aflatoxin by sampling foodstuffs or by dietary questionnaires is extremely imprecise. The development and validation of specific aflatoxin biomarkers represents a significant advance for accurate assessment of exposure in biofluids such as urine and blood.

Concurrent with the early aflatoxin research were a series of studies describing a role for the hepatitis B virus (HBV) in HCC pathogenesis. A number of investigations found that chronic carriers of HBV, as indicated by sequential hepatitis B surface antigen (HBsAg) positivity at 6-month intervals, were at increased risk of developing HCC *(18)*. Further, the age of initial infection was directly related to development of the chronic carrier state and subsequent risk for HCC. Approximately 90% of HBV infections acquired in infancy or early childhood become chronic, whereas only 10% of infections acquired in adulthood become chronic, and less than 50% of chronic carriers progress to HCC *(5, 19–21)*. Finally, the global burden of HBV infection varies geographically and China, Southeast Asia, and sub-Saharan Africa have some of the highest rates of chronic HBV infection in the world, with prevalence of over 10% *(22)*. The public health significance of HBV as a risk factor for HCC is staggering with the consideration that there are over 400 million viral carriers and between 10 and 25% of these individuals are likely to develop HCC *(5, 23, 24)*. The biology, mode of transmission, and epidemiology of this viral infection continues to be actively investigated and has been recently reviewed *(22, 23, 25)*.

To date, the overwhelmingly significant etiological factors associated with development of HCC in the economically developing world are infection in early life with hepatitis B virus (HBV) and lifetime exposure to high levels of aflatoxin B_1 (AFB_1) in the diet *(26, 27)*. Indeed, the multiplicative interaction between HBV and AFB_1 has been documented in two separate cohorts at high risk for HCC *(28–30)*. Over the past 20 years, an appreciation for the role of the hepatitis C virus (HCV) has also emerged. HCV is contributing to HCC being the most rapidly rising solid tumor in the United States and Japan *(31)*. Detailed knowledge of the etiology of HCC

has spurred many mechanistic studies to understand the pathogenesis of this nearly always fatal disease *(2, 26, 32)*.

A number of other environmental exposures have been epidemiologically associated with HCC *(33)*. Vinyl chloride exposure in occupational settings has been associated with the development of HCC in workers and there are now the classic studies associating vinyl chloride exposure with angiosarcomas in the liver *(34–36)*. Recently, studies have found a multiplicative interaction between vinyl chloride exposure in the workplace and alcohol consumption in the enhancement of HCC *(37)*. Finally, a synergistic interaction between vinyl chloride workplace exposure and HBV status has been reported in a cohort in Taiwan *(38)*.

Alcohol is a recognized human carcinogen and has been causally linked to HCC. Alcoholic cirrhosis and heavy alcohol use have been repeatedly associated with an increase in HCC risk *(39)*. However, it is unclear if alcohol use in the absence of cirrhosis influences HCC development *(40)*. Several studies have demonstrated an increased risk of HCC up to 5-fold with consumption of more than 80 g of alcohol per day or approximately 6–7 drinks per day *(39)*. The risk of HCC ranges from borderline significant to doubled with chronic alcohol consumption of less than 80 g/day *(39)*. A synergism between alcohol and HBV and HCV infections has also been described *(39, 41)*. In addition to the association of alcohol and HCC, in economically developed countries the dramatic rise in obesity and nonalcoholic fatty liver disease has also been related to increased HCC *(42–44)*.

Cigarette smoke is a recognized human carcinogen, however, a causal role in HCC is unclear *(45)*. A recent hospital-based case–control study in Italy found no independent effect for tobacco and HCC risk *(46)*. However, a composite analysis of tobacco exposure and cancer risk consistently shows a risk for liver cancer and smoking *(47)*. Finally, the role of hormones in the development of HCC is unclear; however, in some studies, an increased risk of HCC was observed among users of oral contraceptives *(48–50)*. Collectively, these hormonal-related increases in HCC are only seen in low incident countries where exposures to the other major risk factors for this cancer are rare.

4. METHODS FOR BIOMARKER MEASUREMENT

In the case of AFB_1, the measurement of the DNA and protein adducts were of major interest because they are direct products of (or surrogate markers for) damage to a critical cellular macromolecular target. The chemical structures and metabolic pathways leading to the formation of the major aflatoxin macromolecular DNA and protein adducts were known (Fig. 2) *(51, 52)*. The finding that the major aflatoxin–nucleic acid adduct AFB_1–N^7-Gua was excreted exclusively in urine of exposed rats spurred interest in using this metabolite as a biomarker of both exposure and risk. This

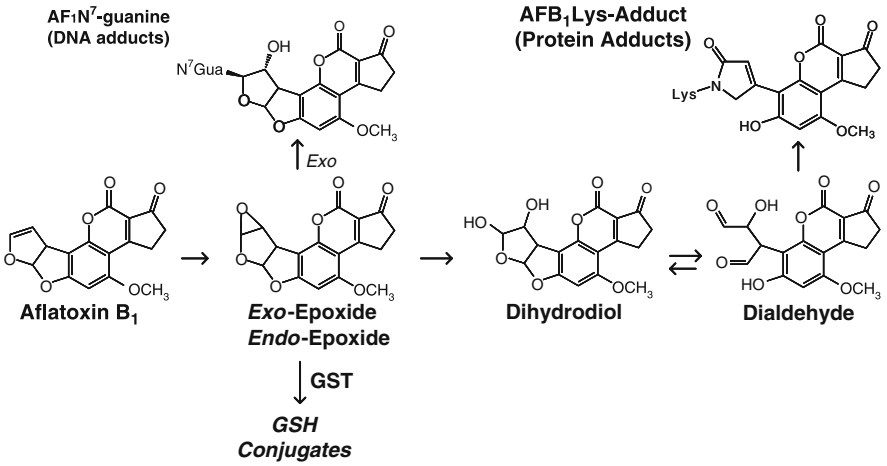

Fig. 2. Structures of aflatoxin biomarkers.

adjunct, however, has a short half-life in the body (~8 h) *(53)*. The serum aflatoxin–albumin adduct was also examined as a biomarker since the longer half-life of albumin (~3 weeks) integrates exposures over longer time periods. Studies in experimental models found that the formation of aflatoxin–DNA adducts in liver, excretion of the urinary aflatoxin–nucleic acid adduct, and formation of the serum albumin adduct were highly correlated *(54)*.

Many different analytical methods were available for quantitation of chemical adducts in biological samples *(55–57)*. Each methodology has unique specificity and sensitivity and, depending on the application, the user can choose which is most appropriate. For example, to measure a single aflatoxin metabolite, a chromatographic method can resolve mixtures of aflatoxins into individual compounds, providing that the extraction procedure does not introduce large amounts of interfering chemicals. Antibody-based methods were often more sensitive than chromatography, but immunoassays are less selective because the antibody may cross-react with multiple metabolites. A recent inter-laboratory collaboration used identical serum sample sets to analyze for aflatoxin–albumin adducts by ELISA, high-performance liquid chromatography (HPLC) with fluorescence detection (HPLC-f), and HPLC with isotope-dilution mass spectrometry (IDMS). Overall, this study showed an excellent correlation between these three independent methodologies conducted in different laboratories *(58)*.

An immunoaffinity cleanup/HPLC procedure was developed to isolate and measure aflatoxin metabolites in biological samples *(59–61)*. With this approach, we performed initial validation studies for the dose-dependent excretion of urinary aflatoxin biomarkers in rats after a single

exposure to AFB_1 *(62)*. A linear relationship was found between AFB_1 dose and excretion of the AFB–N^7-Gua adduct in urine over the initial 24 h period of exposure. In contrast, excretion of other oxidative metabolites, such as AFP_1 showed no linear association with dose. Subsequent studies in rodents that assessed the formation of aflatoxin macromolecular adducts after chronic administration also supported the use of DNA and protein adducts as molecular measures of exposure *(63, 64)*. Recent studies using isotope-dilution mass spectrometry with liquid chromatography separation have demonstrated an increase in sensitivity of at least 1,000-fold over technologies used for the detection of aflatoxin biomarkers 15 years ago *(65–67)*. Further, repeated analysis of serum collected in 1983 from aflatoxin-exposed people has demonstrated that the aflatoxin–lysine adduct in albumin is stable under a range of temperature storage conditions *(68)*.

An area of considerable importance, that has received far less attention than it should, has been in the area of internal standard development. All quantitative measurements require the use of an internal standard to account for sample to sample variations in the analyte recoveries. In the case of mass spectrometry, internal standards generally employ an isotopically labeled material that is identical to the chemical being measured. Obtaining such isotopically labeled materials requires chemical synthesis, if they are not commercially available, and has impeded the application of internal standards in many studies. In the case of immunoassays, internal standards pose a different challenge since the addition of an internal standard that is recognized by an antibody results in a positive value contribution. The dynamic range is usually less than 100 in immunoassays, and therefore great care must be taken to spike a sample with an internal standard so one can obtain a valid result. In contrast, most chromatographic methods result in dynamic ranges of analyses that can be over a 10,000-fold range of levels. The mass spectrometry methods are not only applicable for the quantitation of small molecules such as aflatoxin, but it has also been extended for use to measure mutations in DNA fragments found circulating in plasma that are mechanistically linked to the etiopathogenesis of HCC, such as p53 *(69–72)*.

5. VALIDATION OF BIOMARKERS OF ENVIRONMENTAL CARCINOGENS

In the early 1980s studies to identify effective chemoprevention strategies for aflatoxin carcinogenesis was initiated. The hypothesis was that reduction of aflatoxin–DNA adduct levels by chemopreventive agents would be mechanistically related to and therefore predictive of cancer preventive efficacy. Preliminary data with a variety of established chemopreventive agents demonstrated that after a single dose of aflatoxin, levels of DNA adducts

were reduced *(73)*. A more comprehensive study using multiple doses of aflatoxin and the chemopreventive agent, ethoxyquin, was carried out to examine the relationships between levels and rates of DNA adduct formation and removal and hepatic tumorigenesis in rats. At 3 months after aflatoxin treatment, it was observed that co-treatment with ethoxyquin had reduced both area and volume of liver occupied by presumptive preneoplastic foci by >95%. This same protocol also dramatically reduced binding of AFB_1 to hepatic DNA, from 90% initially to 70% over the course of a 2-week carcinogen-dosing period. Intriguingly, no differences in residual DNA adduct burden, however, were discernible several months after dosing despite the profound reduction in tumor burden.

The experiment was then repeated with several different chemopreventive agents and in all cases aflatoxin-derived DNA and protein adducts were reduced; however, even under optimal conditions, the reduction in the macromolecular adducts always under-represented the magnitude of the diminution in tumor burden *(74, 75)*. These macromolecular adducts can track with disease outcome on a population basis, but in the multistage process of cancer the absolute level of adduct provides only a necessary but insufficient measure of tumor formation.

Experimental validation of the role of human HBV in HCC etiopathogenesis has been compromised by the very restricted nature of the number of species that can become infected with this virus. The chimpanzee and tree shrew can be infected by human HBV but neither has proven to be a cost-effective model for extensive investigation, while the woodchuck and duck can be infected with similar yet distinct HBV strains *(76–78)*. Transgenic mouse models have also been developed that generate a 100% probability of developing HCC *(79)*. These transgenic mice have been used to explore the interaction of the HBV transgene with AFB_1 *(80)*. Collectively, these models are extremely valuable for the study of the underlying molecular pathways in the virally induced cancers but they have to date been of limited value in recapitulating the more complex etiology of human HCC.

Using the chemopreventive agent oltipraz, Roebuck et al. *(74)* established correlations between reductions in levels of AFB_1–N^7-Gua excreted in urine and incidence of HCC in aflatoxin-exposed rats. Overall, reduction in biomarker levels reflected protection against carcinogenesis, but these studies did not address the quantitative relationship between biomarker levels and individual risk. Thus, in a follow-up study, rats dosed with AFB_1 daily for 5 weeks were randomized into three groups: no intervention; delayed-transient intervention with oltipraz during weeks 2 and 3 of exposure; persistent intervention with oltipraz for all 5 weeks of dosing *(81)*. Serial blood samples were collected from each animal at weekly intervals throughout aflatoxin exposure for measurement of aflatoxin–albumin adducts. The integrated level of aflatoxin–albumin adducts over

the exposure period decreased to 20% and 39% in the delayed-transient and persistent oltipraz intervention groups, respectively, as compared with no intervention. Similarly, the total incidence of HCC dropped significantly from 83 to 60% and 48% in these groups. Overall, there was a significant association between integrated biomarker level and risk of HCC. When the predictive value of aflatoxin–serum albumin adducts was assessed within treatment groups, however, there was no association between integrated biomarker levels and risk of HCC. These data clearly demonstrated that levels of the aflatoxin–albumin adducts could predict population-based changes in disease risk, but had no power to identify individuals destined to develop HCC. Because of the multistage process of carcinogenesis, in order to determine individual risk of disease, a panel of biomarkers reflecting different stages will be required.

6. BIOMARKERS IN HUMAN INVESTIGATIONS

Extensive cross-sectional epidemiologic studies have been conducted in high-risk groups for HCC. The HBV biomarkers were developed and validated using the HBsAg biomarker. This work directly led to the research that resulted in a vaccine effective against HBV. Indeed, this vaccine has been reported to reduce HCC in a cohort of young children in Taiwan *(82)*. Further the serology of HBV has been extensively described and developed *(25)*. The work on AFB_1 exposures and its role in HCC etiology has taken a far longer time period to come to fruition. Initial studies in the Philippines *(83)* demonstrated that an oxidative metabolite of aflatoxin could be measured in urine and thus had potential to serve as an internal dose marker. Subsequent work conducted in the People's Republic of China and The Gambia, West Africa, areas with high incidences of HCC, determined that the levels of urinary aflatoxin biomarkers showed dose-dependent relationships with aflatoxin intake. Gan et al. *(84)* and Wild et al. *(85)* also monitored levels of aflatoxin serum albumin adducts and observed a highly significant association between intake of aflatoxin and level of adduct. Many of the aflatoxin studies used different analytical methods and therefore the quantitative comparison of different data sets has been extremely problematic. However, a recent study compared methods of ELISA and mass spectrometry (MS) and found high correlation between these two methods ($r = 0.856$, $p < 0.0001$) *(66)*.

Biomarker development in HCC has been further advanced by the molecular biological studies on the TP53 tumor suppressor gene, the most common mutated gene detected in human cancer *(86, 87)*. Many studies of *p53* mutations in HCC occurring in populations exposed to high levels of dietary aflatoxin have found high frequencies of guanine to thymine transversions,

with clustering at codon 249 *(88, 89)*. In contrast, no mutations at codon 249 were found in *p53* in HCC from Japan and other areas where there was little exposure to aflatoxin *(90, 91)*. The occurrence of this specific mutation has been mechanistically associated with AFB_1 exposure in experimental models including bacteria *(92)* and through demonstration that aflatoxin-8,9-epoxide could bind to codon 249 of *p53* in a DNA plasmid in vitro *(93)*. Mutational analysis of the *p53* gene in human HepG2 cells and hepatocytes exposed to AFB_1 found preferential induction of the transversion of guanine to thymine in the third position of codon 249 *(94, 95 96, 97)*. In summary, studies of the prevalence of codon 249 mutations in HCC cases from patients in areas of high or low exposure to aflatoxin suggest that a G–T transition at the third base is associated with aflatoxin exposure and in vitro data would seem to support this hypothesis.

Although useful, cross-sectional epidemiological studies have the least power to relate an exposure to disease outcome since these studies only provide a view during a short time frame. Data from the cross-sectional aflatoxin biomarker studies demonstrated short-term dose–response effects for a number of the aflatoxin metabolites, including the major nucleic acid adduct, serum albumin adduct, and AFM_1 This information could then be used in follow-up studies to test a number of hypotheses about risk to individuals having high exposures, the efficacy of exposure remediation, and interventions and mechanisms underlying susceptibility.

Longitudinal studies are extremely important in the development and validation process for biomarkers. These investigations permit an understanding of the stability in storage and the tracking potential of each biomarker, which are essential for the evaluation of the predictive power of the biomarker. While long-term stability of many of the HBV markers have been well-established *(98)*, we needed to know whether the aflatoxin metabolites were stable over the long term. The stability of aflatoxin biomarkers was monitored by supplementing urine samples with aflatoxins at the time of collection and then analyzing repeated samples over the course of 8 years. Similarly, aflatoxin–albumin adducts, as described above, in human sera were found to be stable for at least 15 years when stored at –20°C *(68)*. Therefore, at least for some of the aflatoxin biomarkers, degradation over time was not a major problem; however, similar studies are required for all chemical-specific biomarkers.

An objective in development of any biomarker is to use them as predictors of past and future exposure status in people. This concept is embodied in the principle of tracking, which is an index of how well an individual's biomarker remains positioned in a rank order relative to other individuals in a group over time. Tracking within a group of individuals is expressed by the intraclass correlation coefficient. When the intraclass correlation coefficient is 1.0, a person's relative position, independent of exposure, within the group

does not change over time. If the intraclass correlation coefficient is 0.0, there is random positioning of the individual's biomarker level relative to the others in the group throughout the time period. The tracking concept is central to interpreting data related to exposure and biomarker levels and requires acquisition of repeated samples from subjects. Unfortunately, data on the temporal patterns of formation and persistence of aflatoxin macromolecular adducts in human samples are very limited. Obviously, chemical-specific biomarkers measured in cross-sectional studies cannot provide information on the predictive value or tracking of an individual's marker level over time. In contrast to the aflatoxin situation, the HBV biomarker tracking has been well characterized and forms the basis for defining chronic infection status *(98)*.

Tracking is important in assessing exposure and this information is essential in the design of intervention studies. In all these situations it is critical to know how many biomarker samples are required and when they should be obtained. For example, if exposure remains constant and the tracking value for a marker changes over time, it might be assumed that the change in tracking is due to a biological process, such as an alteration in the balance of metabolic pathways responsible for adduct formation. On the other hand, lack of tracking can be attributable to great variance in exposure. Therefore, to determine unequivocally the contributions of intra- and interindividual variations to biomarker levels, experiments must assess tracking over time.

Many published case–control studies have examined the relation of aflatoxin exposure and HCC. Compared with cohort studies, case–control studies are both cost- and time-effective. Unfortunately, case–control studies are often initiated long after exposure has occurred and it cannot be assumed that the exposure has not appreciably changed over time. Also, such studies involve assumptions in the selection of controls, including that the disease state does not alter metabolism of aflatoxin. Thus, matching of cases and controls in a specific biomarker study is much more difficult than in a case–control study involving genetic markers.

One of the first case–control studies compared the dietary intake of aflatoxin in cases of HCC in the Philippines with intake in age- and sex-matched controls. Bulatao-Jayme et al. *(99)* found that the mean aflatoxin exposure per day in cases of HCC was 4.5 times higher than in the controls; however, alcohol consumption was a confounder in this study that may have enhanced this effect. In the Guangxi Autonomous Region of China *(100, 101)* the interaction between HBV infection and dietary aflatoxin exposure dichotomized for heavy and light contamination was examined. Those individuals who were positive for HBsAg and had heavy aflatoxin exposure had an incidence of HCC l0-fold higher than did people living in areas with light aflatoxin contamination *(100)*. In a case–control study in Taiwan, two biomarkers, aflatoxin–albumin adducts and aflatoxin–DNA

adducts in liver tissue samples, were measured *(102)*. The proportion of subjects with a detectable level of aflatoxins–albumin adducts was higher for cases of HCC than for matched controls (odds ratio 1.5). There was also a statistically significant association between detectable level of aflatoxin–albumin adduct and risk of HCC among men younger than 52 years old (multivariant adjusted odds ratio 5.3). Although a number of negative case–control studies of aflatoxin and HCC have been reported *(15)*, the overwhelming evidence from many investigations pointed to an etiological role for aflatoxin in human HCC.

Data obtained from cohort studies have the greatest power to determine a true relationship between an exposure and disease outcome because one starts with a healthy cohort, obtains biomarker samples, and then follows the cohort until significant numbers of cases are obtained. A nested study within the cohort can then be designed to match cases and controls. An advantage of this method is causation can be established (due to the longitudinal nature of cohort studies, there is no temporal ambiguity) and selection bias is minimized. A major disadvantage, however, is the time needed in follow-up (often years) to accrue the cases, especially for chronic diseases such as HCC. This disadvantage can be overcome in part by enrolling large numbers of people (often tens of thousands) to ensure case accrual at a reasonable rate.

To date two major cohort studies with aflatoxin biomarkers have demonstrated the important role of this carcinogen in the etiology of HCC. The first study, comprising more than 18,000 men in Shanghai, examined the interaction of HBV and aflatoxin biomarkers as independent and interactive risk factor for HCC. The nested case–control data revealed a statistically significant increase in the adjusted relative risk (RR) of 3.4 [95% CI: 1.1.–10.0] for those HCC cases where urinary aflatoxin biomarkers were detected. For HBsAg-positive people only the RR was 7 [95% CI: 2.2.–22.4], but for individuals with both urinary aflatoxins and positive HBsAg status the RR was 59 [95% CI: 16.6.–212.0] *(103, 104)*. These results strongly support a causal relationship between the presence of the chemical and viral-specific biomarkers and the risk of HCC.

Subsequent cohort studies in Taiwan have substantially confirmed the results from the Shanghai investigation. Wang et al. *(105)* examined HCC cases and controls nested within a cohort and found that in HBV-infected people there was an adjusted odds ratio of 2.8 [95% CI] for detectable compared with non-detectable aflatoxin–albumin adducts and 5.5 [95% CI] for high compared with low levels of aflatoxin metabolites in urine. In a follow-up study, there was a dose–response relationship between urinary AFM_1 levels and risk of HCC in chronic HBV carriers. Similar to the Shanghai study, the HCC risk associated with AFB_1 exposure was more striking among the HBV carriers with detectable AFB_1–N^7-gua in urine.

Many studies across the globe have explored the relationship between HBV infection and HCC and the risk estimates range from 3 to 30 in case–control studies and from 5 to 148 in cohort studies *(49)*. In the nested case–control study cited above the risk of HCC was 7.3 times higher among HBsAg-positive individuals compared to HBsAg-negative individuals, controlled for smoking and aflatoxin exposures *(29)*. A small hospital-based case–control study from northeast Thailand showed an adjusted odds ratio (OR) of 15.2 for the presence of HBsAg among HCC patients *(106)*. An adjusted OR of 13.5 was reported from a case–control study in The Gambia *(22)*. The risk of HCC among HBsAg positive individuals in Korea from a prospective cohort study of government workers was 24.3 among men and 54.4 among women, adjusted for age, smoking, alcohol use, and diabetes *(107)*. A similar prospective study from Taiwan found men positive for HBsAg were 223 times more likely to develop HCC than men with HBsAg negative *(20)*.

The contribution of HBV to the pathogenesis of liver cancer is multifactorial and is complicated by the identification of mutant variants in HBV that modulate the carcinogenesis process *(108, 109)*. The HBV genome encodes its essential genes with overlapping open-reading frames; therefore, a mutation in the HBV genome can alter the expression of multiple proteins. In many cases of HCC in China and Africa a double mutation in the HBV genome, an adenine to thymine transversion at nucleotide 1762 and a guanine to adenine transition at nucleotide 1764 ($1762^{T}/1764^{A}$), has been found in tumors *(110–112)*. This segment of the HBV genome contains an overlapping sequence for the base core promoter and the HBV X gene; therefore, the double mutation in codon 130 and 131 of the HBV X gene reported in human HCC is identical to the 1762 and 1764 nucleotide changes *(113)*. The increasing occurrence of these mutations have been also associated with the increasing severity of the HBV infection and cirrhosis *(111, 112)*. This acquired mutation following HBV integration into hepatocytes was originally characterized in HBV e antigen negative people *(114)*. The $1762^{T}/1764^{A}$ double mutation occurs more frequently in people infected with the genotype C strains of HBV, which is the most common genotype found in East Asian patients *(115–117)*. This double mutation tracks with an increased inflammatory response that becomes stronger as the progression of liver damage transits through chronic hepatitis and into a cirrhosis stage *(118)*. The underlying mechanism of the effects of HBV e antigen on the biology of inflammation and cirrhosis are still unclear, but there are substantial data that point to modulation of the immune surveillance system and immune tolerance in the presence and absence of this protein *(118–120)*. The $1762^{T}/1764^{A}$ double mutation has also been demonstrated to affect an increase in the rate of HBV genome synthesis in cellular models *(108, 109)*. In cellular studies the $1762^{T}/1764^{A}$ double mutation increased the replica-

tion of the viral genome 2-fold and in the case of some of the rarer triple mutations, an 8-fold increase in genome replication was found *(108, 120)*. Recent data have also shown that there is a sequential accumulation of these mutations in people during the course of the progression to cancer *(121)*.

7. INTERVENTION TRIALS USING AFLATOXIN BIOMARKERS

Clinical trials and other interventions are designed to translate findings from human and experimental investigations to public health prevention. Both primary (to reduce exposure) and secondary (to alter metabolism and deposition) interventions can use specific biomarkers as endpoints of efficacy. Such biomarkers can be applied to the preselection of exposed individuals for study cohorts, thereby reducing study size requirements. They can also serve as short-term modifiable endpoints *(122)*. In a primary prevention trial the goal is to reduce exposure to aflatoxins in the diet. Interventions can range from attempting to lower mold growth in harvested crops to using trapping agents that block the uptake of ingested aflatoxins. In secondary prevention trials one goal is to modulate the metabolism of ingested aflatoxin to enhance detoxification processes, thereby reducing formation of DNA adducts and enhancing elimination.

The use of aflatoxin biomarkers as efficacy endpoints in primary prevention trials in West Africa has been recently reported *(123)*. This study assesses postharvest measures to restrict aflatoxin contamination of groundnut crops. Six hundred people were monitored and in control villages mean aflatoxin–albumin concentration increased postharvest (from 5.5 pg/mg [95% CI: 4.7–6.1] immediately after harvest to 18.7 pg/mg [17.0–20.6] 5 months later). By contrast, mean aflatoxin–albumin concentration in intervention villages after 5 months of groundnut storage was much the same as that immediately postharvest (7.2 pg/mg [6.2–8.4] vs. 8.0 pg/mg [7.0–9.2]). At 5 months, mean adduct concentration in intervention villages was less than 50% of that in control villages (8.0 pg/mg [7.2–9.2] vs. 18.7 pg/mg [17.0–20.6], $p < 0.0001$). Thus, primary prevention may be an effective means to reduce HCC burden, especially in areas where single foodstuffs such as groundnuts are major components of the diet.

Aflatoxin biomarkers were also used as intermediate biomarkers in a Phase IIa chemoprevention trial of oltipraz in Qidong, PRC *(124–126)*. This was a placebo-controlled, double-masked study in which participants were randomized to receive placebo or 125 mg oltipraz daily or 500 mg oltipraz weekly. Urinary AFM_1 levels were reduced by 51% compared with the placebo group in persons receiving the 500 mg weekly dose. No significant differences were seen in urinary AFM_1 levels in the 125 mg group compared with placebo. This effect at the higher dose was thought to be

due to inhibition of cytochrome P450 1A2 activity. Median levels of AFB_1–mercapturic acid (a glutathione conjugate derivative) were elevated 2.6-fold in the 125 mg group, but were unchanged in the 500 mg group. Increased AFB_1–mercapturic acid reflects induction of aflatoxin conjugation through the actions of glutathione S-transferases. The apparent lack of induction in the 500 mg group probably reflects masking due to diminished substrate formation for conjugation through the inhibition of CYPlA2 seen in this group.

This strategy was extended to chlorophyllin, an anticarcinogen in experimental models when given in large molar excess relative to the carcinogen at or around the time of carcinogen exposure. Chlorophyllin cuts by forming molecular complexes with carcinogens such as aflatoxin in the gastrointestinal tract, thereby blocking bioavailability. One hundred eighty healthy adults from Qidong were randomly assigned to ingest 100 mg chlorophyllin or a placebo three times a day for 4 months. The primary endpoint was modulation of levels of aflatoxin–N^7-guanine adducts in urine samples collected 3 months into the intervention measured using sequential immunoaffinity chromatography and liquid chromatography–electrospray mass spectrometry. Chlorophyllin consumption at each meal led to an overall 55% reduction in median urinary levels of this aflatoxin biomarker compared to those taking placebo *(127)*. Recently, we tested whether drinking hot water infusions of 3-day-old broccoli sprouts, containing defined concentrations of glucosinolates as a stable precursor of the anticarcinogen sulforaphane, could alter the disposition of aflatoxin. Sulforaphane, like oltipraz, acts to increase expression of aflatoxin detoxication enzymes in the liver and other tissues. Two hundred healthy adults drank infusions containing either 400 or < 3 μmol glucoraphanin nightly for 2 weeks. Urinary levels of AFB_1–N^7-Gua were not different between the two intervention arms. However, measurement of urinary levels of dithiocarbamates (sulforaphane metabolites) indicated striking interindividual differences in bioavailability. Presumptively, there were individual differences in the rates of hydrolysis of glucoraphanin to sulforaphane by the intestinal microflora of the study participants. Nonetheless, an inverse association was observed for excretion of dithiocarbamates and aflatoxin–DNA adducts in individuals receiving broccoli sprout glucosinolates *(128)*.

8. DNA MUTATIONS MEASURED IN HUMAN PLASMA AND HCC

The development and validation of biomarkers for early detection of disease or for the identification of high-risk individuals is a major translational effort in cancer research. α-Fetoprotein is widely used as a HCC diagnostic marker in high-risk areas because of its ease of use and low cost. *(129)*

However, this marker suffers from low specificity due to its occurrence in diseases other than liver cancer. Moreover, no survival advantage is seen in populations when α-fetoprotein is used in large-scale screening *(130)*. Such inadequacies have contributed to the need to identify other molecular biomarkers that are possibly more mechanistically associated with HCC development, including hypermethylation of the p16 gene, p15 gene, GSTP1 promoter regions, and codon 249 mutations in the p53 gene *(131–134)*. Results from investigations of p16, p15, GSTP1 promoter hypermethylation, and p53 mutations indicate that these markers are prevalent in HCC, but there is as of yet limited information on the temporality of these genetic changes prior to clinical diagnosis.

Several studies have now demonstrated that DNA isolated from serum and plasma of cancer patients contains the same genetic aberrations as DNA isolated from an individual's tumor *(70, 135, 136)*. The process by which tumor DNA is released into circulating blood is unclear but may result from accelerated necrosis, apoptosis, or other processes *(137)*. While the detection of specific p53 mutations in liver tumors has provided insight into the etiology of certain liver cancers, the application of these specific mutations to the early detection of cancer offers great promise for prevention *(138)*. In a seminal report, Kirk et al. *(139)* reported the detection of codon 249 p53 mutations in the plasma of liver tumor patients from The Gambia; however, the mutational status of the tumors was not known. These authors also reported a small number of cirrhosis patients having this mutation and given the strong relation between cirrhosis and future development of HCC, raised the possibility of this mutation being an early detection marker. Jackson et al. *(140)* used short oligonucleotide mass analysis (SOMA) in lieu of DNA sequencing for analysis of specific p53 mutations in HCC samples. Analysis of 20 plasma and tumor pairs showed 11 tumors containing the specific mutation, 6 of the paired plasma samples exhibited the same mutation.

The temporality of the detection of this mutation in plasma before and after the clinical diagnosis of HCC was facilitated by the availability of longitudinally collected plasma samples from a cohort of 1,638 high-risk individuals in Qidong, PRC, that have been followed since 1992 *(141)*. The results showed that in samples collected prior to liver cancer diagnosis, 21.7% of the plasma samples had detectable levels of the codon 249 mutation. The persistence of this prediagnosis marker was borderline statistically significant. The codon 249 mutation in p53 was detected in 44.6% of all plasma samples following the diagnosis of HCC. Collectively these data suggest that nearly one half of the potential patients with this marker can be detected at least 1 year and in 1 case 5 years prior to diagnosis.

Using a novel internal standard plasmid, plasma concentrations of p53 codon 249-mutated DNA were quantified by SOMA in 89 hepatocellular carcinoma cases, 42 cirrhotic patients, and 131 nonliver diseased control

subjects, all from highly aflatoxin-exposed regions of The Gambia *(72)*. The hepatocellular carcinoma cases had higher median plasma concentrations of the p53 mutation (2,800 copies/mL; interquartile range: 500–11,000) compared with either cirrhotic (500 copies/mL; interquartile range: 500–2,600) or control subjects (500 copies/mL; interquartile range: 500–2,000). Levels of >10,000 copies of p 53 codon 249 mutation/mL plasma were also significantly associated with the diagnosis of HCC (odds ratio, 15; 95% confidence interval, 1.6–140) when compared with cirrhotic patients. Potential applications for the quantification of this alteration of DNA in plasma include estimation of long-term, cumulative aflatoxin exposure and selection of appropriate high-risk individuals for targeted intervention.

In many cases of HCC in China and Africa a double mutation in the HBV genome, an adenine to thymine transversion at nucleotide 1762 and a guanine to adenine transition at nucleotide 1764 (1762T/1764A), has been found in tumors *(142, 143)*. Kuang et al. *(144)* examined, with mass spectrometry, the temporality of an HBV 1762T/1764A double mutation in plasma and tumors. Initial studies found 52 of 70 (74.3%) tumors from Qidong, PRC contained this HBV mutation. Paired plasma samples were available for six of the tumor specimens; four tumors had the HBV 1762T/1764A mutation while three of the paired plasma samples were also positive. The potential predictive value of this biomarker was explored using stored plasma samples from a study of 120 residents of Qidong who had been monitored for aflatoxin exposure and HBV infection. After 10 years passive follow–up, there were six cases of major liver disease and all had detectable levels of the HBV 1762T/1764A mutation up to 8 years prior to diagnosis. Finally, 15 liver cancers were selected from a prospective cohort of 1,638 high-risk individuals in Qidong and the HBV 1762T/1764A mutation was detected in 8 of the 15 cases prior to cancer. The persistence of detection of this mutation was statistically significant. We have therefore found that a prediagnosis biomarker of specific HBV mutations can be measured in plasma and suggest this marker for use as an intermediate endpoint in prevention and intervention trials.

9. SUMMARY

HCC is a slowly developing process involving progressive genetic insults and their resulting genomic changes *(145, 146)*. HCC may not become evident until over 30 years after chronic infection with HBV, HCV, and/or aflatoxin exposure. Chronic hepatitis and cirrhosis may only develop 5 years before HCC is evident and globally, 70–75% of all HCC is accompanied by cirrhosis *(110, 145)*. This genomic heterogeneity may be a reflection of the different etiologies of HCC and their effect upon the molecular regulation of

hepatocytes *(146)*. Over the past 25 years, the development and application of molecular biomarkers reflecting events from exposure to manifestation of clinical diseases has rapidly expanded our knowledge of the mechanisms of HCC pathogenesis. These biomarkers will have increasing potential for early detection, treatment, and prevention.

The molecular epidemiology investigations of aflatoxin, HBV, and HCC probably represent one of the most extensive data sets in the field of environmental carcinogenesis and this work may serve as a template for future studies of the role of other environmental agents in human diseases with chronic, multifactorial etiologies (Fig. 3). The development of these biomarkers has been based upon the knowledge of the biochemistry and toxicology of aflatoxins gleaned from both experimental and human studies. These biomarkers have subsequently been utilized in experimental models to provide data on the modulation of these markers under different situations of disease risk. This systematic approach provides encouragement for design and successful implementation of preventive interventions.

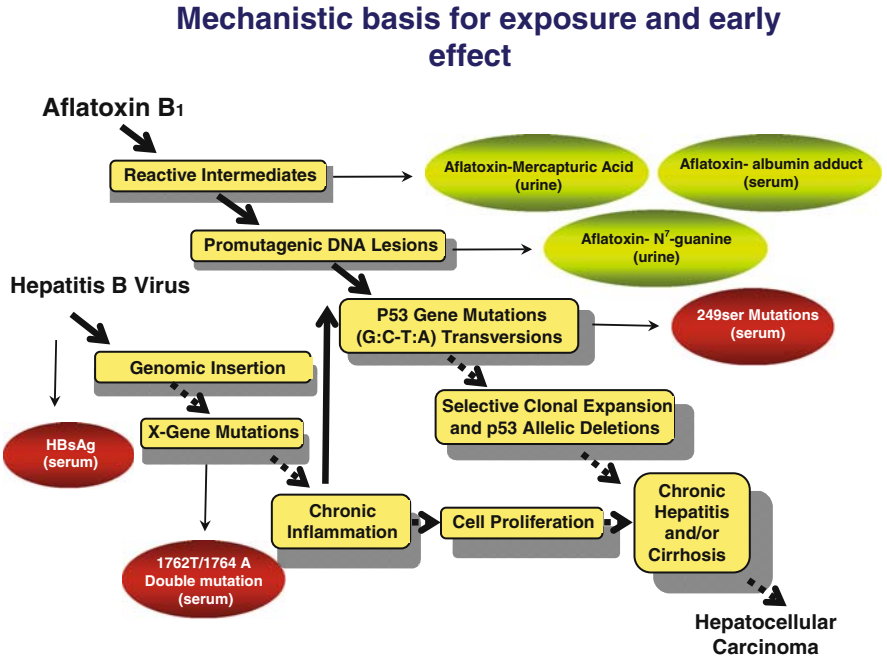

Fig. 3. Mechanistic-based biomarkers of aflatoxin and HBV.

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3 Primary Liver Cancer: Chemical Carcinogenesis

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CONTENTS

ABSTRACT

Liver cancer is an important form of cancer worldwide ranking in the top ten in both incidence and mortality *(1)*. Over 200,000 new cases of primary hepatocellular carcinoma are diagnosed worldwide each year *(1)*. The American Cancer Society predicts over 22,000 new cases of liver and bile duct cancer and that nearly 18,000 individuals will die of this disease in the year 2009 *(2)*. In the United States and Europe, primary liver cancer is fairly rare, but in some parts of the world, it is the primary type of cancer observed *(1)*. Environmental influences, including carcinogen exposure, are believed to contribute to its distinct geographical distribution pattern *(3)*. Although rare genetic disorders can contribute to liver cancer development, ethanol and dietary factors are known to contribute to its incidence and progression *(3)*.

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The prevalence of liver cancer and its high mortality rate indicates the need for appropriate animal models of this disease in order to develop treatment and intervention strategies. In addition, the liver is the primary site for cancer induction in the bioassays used for carcinogen testing indicating the necessity for extrapolation of neoplasms that arise at this site in animals to man. The utility of defining common biomarkers for the conversion of benign to malignant transition will assist in developing appropriate inter-species extrapolation for risk assessment. The inclusion of early lesions from preclinical models will permit assessment of the ability of methods to develop appropriate risk assessment. In addition, analysis of liver cancer development is a useful model for study of the carcinogenic process of solid tumors that arise in both humans and animals. The influence of genetic background and environmental factors on neoplastic development is readily studied in rodent models of this disease.

While genetic factors can contribute to primary liver cancer development, environmental factors have an important role in human liver cancer development. The liver is exposed to ingested materials and has a high level of metabolism. The liver is susceptible to liver cancer development by chemicals and rodent liver has been used as a model to understand the role that chemicals play in liver cancer development and progression. In the human, cirrhosis is an important contributor to most primary liver cancer development. Viral hepatitis can lead to cirrhosis and certain chemical exposures to contribute to this baseline liver disease and can exacerbate the potential for liver cancer. These include aflatoxin, ethanol, and potentially other dietary constituents (limited antioxidant intake (selenium, Vitamin E), iron excess, and others). Ethanol and NASH can contribute to the development of cirrhosis and likewise can lead to HCC development. Chemicals that can increase the incidence of neoplasms in animals can be classified into genotoxic and nongenotoxic modes of action. The effects of agents with a carcinogenic potential are dose dependent. Understanding the biological basis of the changes that occur during the cancer induction and progression process, as well as the changes that chemicals induce in the liver will improve our knowledge of the steps and stages in the pathogenesis of primary liver cancer.

Key Words: Chemical carcinogens; primary liver cancer; HCC; genotoxins; nongenotoxins

1. INTRODUCTION

The biology of the liver, the biological processes involved in cancer development, and the etiological factors involved in liver cancer development provide a focus on the early processes and signaling pathways important

in primary liver cancer development. Perhaps, the most important point to consider is the cell population at risk for initiation of the cancer process in the liver. Since most hepatocytes are in Go phase, first proliferation must be stimulated. Under normal conditions, single cell death is followed by replacement of that hepatocyte. One hypothesis is that cancer stem cells are bipotential and can be stimulated to proliferate *(4)*. Their (oval cells) outgrowth can occur under situations where a large percentage of the liver is damaged. The stem cells then differentiate into hepatocytes or cholangiocytes depending on the degree and duration of damage. Agents that cause extensive damage to the liver can result in neoplastic changes that are fetal in nature. A second hypothesis is that mature hepatocytes are the cell population at risk for early preneoplastic changes *(5)*. Mature hepatocytes can develop into focal areas of proliferation that in turn can become nodular areas of hyperplasia. In this case, both poorly differentiated, small cell lesions (that are primarily diploid) and large cell, more highly differentiated (tetraploid or higher ploidy) lesions develop *(6)*. Understanding the etiology, proliferative and differentiation cues for the liver, and the mechanisms of the carcinogenesis process in the liver is key to understanding the role of chemicals in the development of HCC (Table 1).

Chemical, biologic, and physical agents can contribute to cancer development. Perturbations in single cells lead to the focal outgrowth of putatively preneoplastic lesions. The altered areas can evolve into nodular hyperplasia, focus in nodule pathology, and areas of frank malignancy *(6)*. To determine the contributions of chemicals to the carcinogenic process in the liver, a variety of animal models have been developed. Since the liver is the primary site for cancer induction in the bioassays used for carcinogen testing, there is a need for extrapolation of animal neoplasms that arise at this site to man. The utility of defining common biomarkers for the conversion of benign to malignant transition will assist in developing appropriate interspecies extrapolation. The inclusion of early lesions from preclinical models will permit assessment of the early changes that occur prior to the onset of clinically detectable disease to our understanding of HCC.

2. LIVER CANCER IS AN IMPORTANT BIOLOGICAL PROBLEM

Liver cancer is an important form of cancer worldwide ranking in the top ten in both incidence and mortality *(7, 8)*. Hepatocellular carcinoma (HCC) is the primary form of liver cancer. Primary liver cancer is the sixth most common form of cancer (626,000 cases/year) in terms of incidence *(9)*. In addition, it is the third most common cause of death (598,000 deaths/year) from cancer *(10)*, with 80% of cases (and deaths) occurring in developing

Table 1
Different Chemicals and Their Role in Development of HCC

Chemical/toxin	*Mode of action*	*Biological effects*
Aflatoxin B1	AFB1 forms covalent bonds with DNA, binds to free amino groups of amino acids	Carcinogenic, mutagenic and toxic effects
Alcohol abuse and tobacco	Evidence for direct action is not clear. The abuse exacerbates the action of HCV, HBV, cirrhosis-mediated HCC	It is a risk factor for the progression of HCC
Oral contraceptive	Requires chronic exposure (>5 years) to mestranol, ethinyl estradiol. Mechanism not clear	Benign hepatic adenomas. Prolonged use with high doses and potency leads to HCC
Dioxin	Acts via AhR which binds to arnt and ultimately results in overexpression of many anti-apoptotic genes. It also induces many metabolizing enzymes that are responsible for toxic intermediates	Enhances proliferation and inhibit apoptotic processes. Causes increase in the size of the liver and ultimately causes liver damage
Phenobarbital	Mechanism is tightly linked with induction of CYP2B1 and the activation of CAR. Low levels of TGFβ1 and elevated levels of anti-apoptotic Bcl2 have been reported.	PB is a liver tumor promoter in rodents
PPAR agonists	The agonists increase TGFβ1 aiding hepatocarcinogenesis	PPARα agonist produces liver tumors in rodents. It causes hepatomegaly and cell proliferation

countries. Surveillance Epidemiology and End Results (SEER), the National Cancer Institute's statistical unit, estimated that 19,000 new cases of liver and intrahepatic bile duct cancer were diagnosed and nearly 17,000 people died from this disease in the United States in 2007 *(2)*. Understanding the

processes that contribute to the cancer development process are important components of determining how and where certain compounds contribute to liver cancer development and progression. Environmental influences, including carcinogen exposure, are believed to contribute to the distinct geographical distribution pattern of primary liver cancer *(11)*. Another important cause of primary liver cancer in humans is viral with both HCV and HBV infection contributing to its incidence. According to NHANES 3, the number of individuals with chronic HCV infection is greater than 3 million in the part of the US population sampled *(12*; *13)*. Chronic infection with hepatitis C virus (HCV) is known to be a major risk factor for development of hepatocellular carcinoma (HCC). In general, HCC develops only after two or more decades of HCV infection and in those with advanced fibrosis *(14)*. Cirrhosis is also an important factor associated with the development of primary liver cancer and hence is an important control for liver cancer biomarker development, most liver cancer arises in the context of cirrhosis. In the United States, less than 30% of HCC is viral in etiology. Excess alcohol use and diabetes mellitus are independent risk factors for liver cirrhosis and are associated with liver cancer development in the United States *(15)*. In addition, smoking may contribute to the risk of liver cancer development. The residual 10% of attributable risk of HCC may be due to or influenced by hereditary metabolic disease factors (such as hemochromatosis). Although rare genetic disorders can contribute to liver cancer development, ethanol and dietary factors are known to contribute to its incidence and progression *(2*, *3)*. The prevalence of liver cancer and its high mortality rate indicates the need for appropriate animal models of this disease in order to develop treatment and intervention strategies. In addition, the pathogenesis of primary liver cancer development for different etiologies needs to be better delineated. The influence of genetic background and environmental factors on neoplastic development is readily studied in rodent models of this disease.

3. CHEMICAL CARCINOGENS

Carcinogenesis can be induced by physical, biological, or chemical means. Agents that act to increase the incidence of cancer in appropriate organisms compared with concurrent and/or historic controls are considered carcinogens. The identification of a carcinogenic potential for an agent delineates the conditions of exposure (dose, time, and duration) under which the agent may induce cancer. Animals are surrogate models of humans since they possess similar physiology and biochemistry. This similarity is not absolute; hence any hazard detected must be examined in the context of human relevance in order to understand the conditions of exposure that may pose a plausible risk to humans. Each human HCC is detected

at different points along the pathogenesis continuum. Several factors are important for cancer development including a loss of normal growth control with contributions from inhibition of apoptosis and enhanced but altered proliferation control *(16)*. In addition, an altered differentiation status can contribute to cancer development and progression. The morphology and certain aspects of the natural history of rodent and human cancer are coincident although the etiology and the exact molecular pathogenesis may diverge between rodents and man. Although several parallel pathways may be induced, the pathway for cytogenetic alterations observed in a specific cancer type is similar in rats, mice, and men. The latency period between initiation of early precancer changes in a single cell and its selection for malignant growth comprises the reversible stage of tumor promotion. In the human, exposure to dietary contaminants such as aflatoxins, as well as calorie overload, ethanol over use, and methyl deficiency can contribute to the risk of primary liver cancer. Certain metals (iron and copper) have been associated with an increased risk of primary liver cancer. Thus, a number of classes of chemical agents can increase the incidence of hepatic neoplasms depending on their dose and duration of exposure.

3.1. Genotoxic Carcinogens

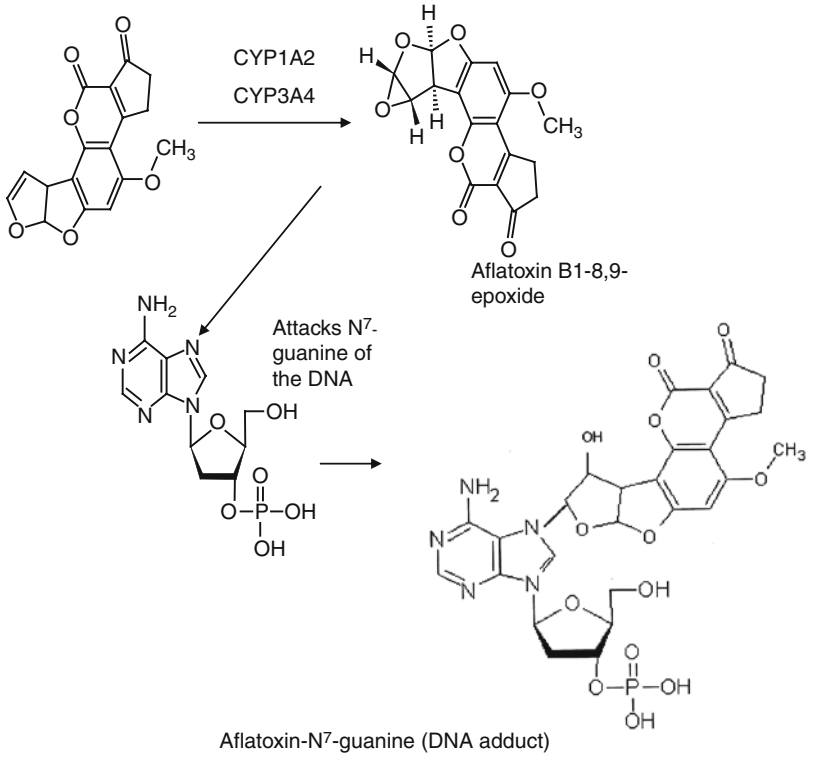
Chemically induced carcinogenesis has been examined experimentally for less than 100 years *(17, 18)*. Initial studies provided the compounds typically in the diet for extended periods of time. For example, the studies of Sasaki and Yoshida *(19)* demonstrated that chemicals could cause hepatic neoplasms in animals. Provision of *o*-aminoazotoluene in the diet led to liver neoplasms in rats. Similarly, Kinoshita *(20)* demonstrated that feeding 4-dimethylaminoazobenzene to rats resulted in liver neoplasms. These findings suggest that agents can be carcinogenic at sites distant from their initial application. Importantly, analogues of these agents have also been examined allowing some structural information to be gathered about the properties of agents that have a carcinogenic potential *(21)*. There is some tissue specificity for carcinogenic action as polycyclic aromatic hydrocarbons are not typically carcinogenic to the liver (except in some circumstances during the neonatal period), while they are to the skin *(22)*. Similarly, certain azo dyes, while carcinogenic to the liver, do not have this activity in the skin *(23)*. The agent 2-acetylaminofluorene but not its related regioisomer, 4-acetylaminofluorene, is carcinogenic in the rodent liver *(24)*. However, dialkyl nitrosamines and several analogs are cytotoxic to the liver and are carcinogenic in rodents and many other mammals *(25)*. These activities are dose dependent and high doses induce acute toxicity, while lower doses are tolerated but can result in neoplasms if the dose and duration of exposure is sufficient. Similarly, aflatoxin produced by the fungus *Aspergillus flavus*

is acutely cytotoxic. This agent is also carcinogenic in all species examined, although the mouse is relatively resistant to its carcinogenic action *(26)*. A variety of other agents in food can also be carcinogenic to the liver including certain mycotoxins *(27)* in addition to aflatoxin (fumonisin in rodents) and pyrrolizidine *(28)* alkaloids (found in comfrey and riddelline). In addition, a dearth of antioxidants and a lack of lipotropes *(29*, *30)* can lead to cancer development in the rodent.

3.1.1. DNA Adducts

This initial class of agents is capable of altering the genetic material either directly, through one of its metabolites, or through perturbation of the processes controlling its actions. Agents that modify the DNA can initiate the carcinogenic process *(31)*. These agents can be metabolized to form DNA adducts or may directly form them. Alternatively, such agents can alter the methylation status of the DNA. In each case, the DNA is modified in a manner that results in heritable changes. In the case of DNA adducts coupled with cell proliferation mutations can result *(32)*. Such mutations can alter the function of selected genes, in some cases inactivating them and in other cases enhancing their activity *(33)*. The dose and duration of exposure of an agent is an important contributing factor to understanding the carcinogenic risk of an agent at doses to which humans are exposed. Many agents with a carcinogenic potential can be metabolized to an electrophilic form. These reactive metabolites can bind to cellular nucleophiles including DNA, RNA, proteins, and lipids *(23)*. The biological consequences of these actions differ. Early studies by James and Elizabeth Miller *(34)* demonstrated that certain carcinogenic agents did not directly bind to proteins, but that following incubation of the compound with tissue extract, the compound or some derivative could be found bound to protein in normal liver but not in the resulting neoplasm. This harbinger of metabolic activation or reactive metabolite formation would lead to the determination that the cell could metabolize some compounds to a reactive form. For example, AAF is metabolized by ring hydroxylation *(35)* and by N-hydroxylation *(36)*. The *N*-hydroxy metabolite can be demonstrated to be more carcinogenic than the parent AAF *(23)*. The *N*-hydroxy AAF is further metabolized by esterification with glucuronyl, acetyl, and sulfate groups. Although conjugation can lead to inactivation of reactive metabolites, in certain cases it can form more reactive agents with facile leaving groups. This is the case for some esters of *N*-hydroxy AAF *(23)*. In addition to the formation of reactive metabolites, certain agents can form free radicals *(37)*. Free radicals have no charge, but have an unpaired electron that makes them reactive. This process can be facilitated by the presence of free iron or copper. Endogenous processes can form free radicals and metabolism of certain carcinogenic agents can also lead to their generation *(38)*. Many agents with a carcinogenic potential can be metabolized to

reactive forms providing a mechanism to understand species differences and individual risks. Understanding the structural basis for metabolic activation permits the prediction of agents that are likely to be directly genotoxic or that can be metabolized to a genotoxic form. In addition, it generates a physicochemical basis for understanding mutagenesis at specific sites in the DNA and in specific tissues. Careful analyses of structures that are positive in rodent bioassays have yielded reactive groups that yield structural alerts for carcinogenic risk *(39, 40)*.

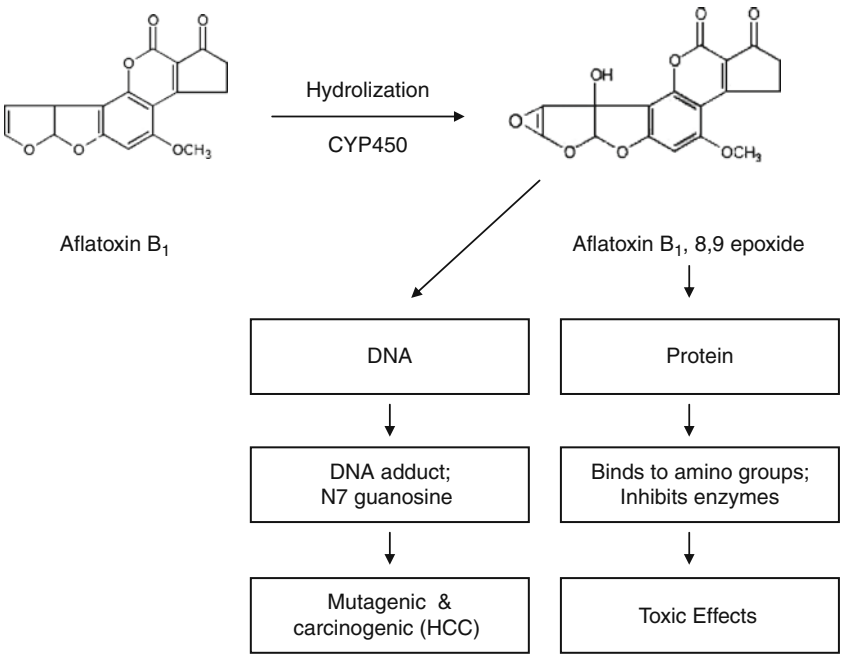


3.1.2. Mutations and Their Consequences

The reaction of electrophilic substances with the DNA results in physicochemical changes in the DNA. The high prevalence of cancer in individuals with an inability to remove DNA adducts in DNA repair deficiencies indicate the important role of DNA damage in cancer induction *(41)*. Similarly, the high incidences of mutations in selected genes in animal models of cancer further demonstrate that DNA damage is the basis of early cancer development *(33)*. Alkylation of DNA can occur by carcinogenic agents that can be metabolized to reactive forms. In this case, the reactive metabolite can covalently adduct to the DNA *(42)*. For example, aflatoxin B1 can be

metabolized to 8,9-epoxide of AFB1, which then binds to N7 guanosine leading to mutations *(43)*. Mutation of G to T can occur at multiple sites, most notably at 249Ser of P53 *(44)*. Methylation, ethylation, and other alkylations can occur with each of the bases as well as the sugar and phosphate backbone *(45*, *46)*. Direct-acting electrophiles can bind to the N7 of guanine, while softer electrophiles can bind to the ring oxygens of the bases. Formation of bulky adducts can occur on the purine ring, while small alkylations can occur more ubiquitously. At lower exposures, selective alkylation can occur, which may or may not be repaired. The presence of DNA adducts and the repair of these lesions can result in mutation. As the adduct burden increases with increased dose/duration of exposure, the repair can be more extensive and over a greater span of the DNA. In addition, as dose/duration increases more cell types may become involved as metabolism shifts and conjugation reserves are depleted. Repair can outpace adduct conversion to mutations under some circumstances. When the lesion is repaired, either the base is removed or a larger segment of DNA is removed. Each of these processes can have different rates and consequences and each is dose dependent.

Point mutations, frameshift mutations, chromosome aberrations, and aneuploidy can occur following chemical administration. Because of the



degree of adduct formation, the site of adduct formation, the ability of adducts to be repaired, and the degree of metabolism to reactive forms, differential activity can be seen in individual cells, tissues, organisms, and species. One consequence of the presence of DNA adducts is cell death. Apoptosis is observed at lower concentrations followed at higher exposures and degrees of damage by necrosis. Direct-acting carcinogens are reactive without requiring metabolic activation and are often carcinogenic at the sites of exposure in multiple species *(47)*. Methylation or ethylation of DNA can lead to base mispairing *(45*, *48)*. Because these simple alkylations are similar to or can result from endogenous processes, they are not as actively repaired. In part, the more persistent DNA adducts/lesions are the ones that have an important mutagenic consequence. For example, ethylating agents can adduct at O6 alkylguanine and O4 alkylthymidine. The O6 adduct is readily repaired, while the O4 adduct is more persistent leading to base mispairing with different consequences for both lesions *(49*, *50)*.

The consequence of bulky adduct presence is to block DNA synthesis resulting in noncoding *(46)*. However, the DNA synthetic machinery can bypass such lesions placing in its stead the most abundant nucleotide, generally an adenine *(51)*. Since bulky adducts are typically at guanines, this is a useful strategy that can, however, result in more marked consequences when more than one base is affected or the adduct was not at guanine. Using 2-AAF as an example, the parent is not mutagenic, but it can be esterified to the sulfate ester that is highly reactive; binding to the N7 of guanine as well as the N3 of guanine *(23)*. In contrast to the formation of a covalent bulky adduct by 2-AAF that distorts the DNA structure, 2-aminofluorene, which also forms bulky adducts at the same sites, sits outside of the helix and does not distort it. As a consequence, 2-AF can induce point mutations, while 2-AAF can lead to frameshift mutations *(52)*. Biological consequence of the presence of DNA adducts is a function of their persistence in the DNA *(53)* and impacts their tissue and species specificity. The persistence of DNA adducts in viable cells has consequences when cell proliferation occurs to fix the mutation before repair can occur *(32)*. Once the mutation is fixed, its location in the genome, the expression of that DNA, and the importance of the affected gene in that stage of the differentiation of the cell, both impact its consequent mutation and the ultimate consequence of a given adduct. Although susceptibility to cancer induction can be modified by polymorphisms in DNA repair genes *(41)*, carcinogen metabolism *(54)*, and immune system *(55)* differences, genes that regulate cell growth and proliferation are more frequently the targets of carcinogens. Both protooncogene and tumor suppressor gene function can be altered by carcinogen exposure *(56–58)*. For example, oncogenes such as Ha-ras can be activated by a single point mutation *(59)*. Activation of Ha-ras is an important mechanism of HCC induction and development in the mouse *(33*, *60)*, but not in rats or humans

(18). In the liver, activation and mutation of β-catenin (and possibly axin) is an important aspect of some types of liver cancer *(61*, *62)*. Similarly, mutations in HNF1 can result in loss of differentiation status as evidenced by loss of expression of a number of drug-metabolizing genes in the neoplasm. Although mutations have been observed in a number of genes in HCC development and progression, only a few genes have non-random mutations. The genes found in HCC that contain mutations include p53, IGF2R, CycD, CycA, BCL10, met, RB, TRα, or β *(6)*. Etiologic agents have been examined with respect to the resulting mutations observed in specific genes including p53, β-catenin, and HNF1. There appear to be multiple pathways that can lead to HCC initiation and progression *(62)*.

Endogenous DNA modifications can be perturbed and this perturbation can contribute to chemical carcinogenesis. Hydroxylation of DNA bases can also occur both through endogenous processes and by certain DNA damaging agents *(63)*. Repair processes for oxidative damage are pervasive in most cell types nonetheless oxidized bases can persist *(64)*. Although all of the DNA bases can be oxidized, the most common are 8-hydroxy deoxyguanosine *(65)* and 5-hydroxymethylthymine *(66)*. These oxidative bases likely arise through endogenous processes *(67)* and they are readily repaired. The most prevalent endogenous modification of DNA is methylation of deoxycytidine *(68*, *69)*. Chemical carcinogens can perturb this process by adduct formation, altered one-carbon pools, single strand break formation, or inactivation of the enzymes involved in the methylation process *(70)*. Diets deficient in lipotropes can result in marked steatosis followed in time by HCC formation in rodents *(30)*. Methyl-deficient diets can result in DNA hypomethylation. Global hypomethylation results in re-expression of genes in general, while hypermethylation results in their silencing *(71)*. Perturbation of nucleosomes, minor and major groove protein binding, and the DNA repair process can likewise lead to DNA perturbations. The presence of a DNA adduct does not mean that a mutation will occur, but it does increase the probability.

3.1.3. The Role of Cell Proliferation in Cancer Initiation

The presence of DNA adducts coupled with cell proliferation can lead to mutation. This process is called fixation wherein the mutation is fixed when an adduct or other DNA alteration persists through a cycle of DNA synthesis *(32)*. Thus, the rate of cell proliferation and DNA synthesis can impact DNA damage *(72)*. In situations where repair processes are normal, high rates of cell proliferation can still lead to mutations. Inherited defects in DNA repair lead to an increased risk of neoplasia *(46)* in many cell types especially in the GI tract with its high rate of exposure to potentially mutagenic agents and its high rate of proliferation. Hepatocytes turn over slowly by comparison

except in circumstances of persistent inflammation induced by hepatitis (viral, alcohol, or drug induced). DNA polymerases are not completely faithful in their replication of the DNA *(73, 74)*. Since a variety of types of DNA damage can occur, many processes exist to remedy their activity. Excision repair can remove either a modified base or nucleotide. The presence of an adduct will result in excision and repair with more bases removed and potentially misrepaired for nucleotide excision compared with base excision repair. Single strand breaks are readily repaired. The repair of double strand breaks is more problematic *(75)* and a nonhomologous end joining process is used that is error prone *(76)*. Mismatch repair can occur when bases are mispaired or when it appears that they are mispaired due to the presence of a DNA modification *(77)*. Perturbation of the mismatch repair process can result in mutations. Larger DNA damage including amplifications, deletions, and aneuploidy can occur. Agents that lead to these lesions contribute to the carcinogenesis process by altering gene dosage of critical genes and/or perturbing their expression. Although mutations alone do not lead directly to neoplasia, they can contribute to the process when they occur in genes critical for cell survival, proliferation, apoptosis, and differentiation status.

3.2. Non-Genotoxic Mechanisms of Chemical Carcinogenesis

A variety of compounds other than mutagenic agents can contribute to liver cancer development. These agents have in common the ability to alter cell survival either by increasing cell proliferation or decreasing apoptosis. Agents that have this activity include those that cause cytotoxicity and those that perturb signaling pathways associated with growth factors, some of which act through nuclear receptors *(18, 78)*. Certain agents are cytotoxic at either high doses or with chronic administration *(79)*. These agents such as chloroform do not pose a risk when exposure occurs below the threshold for cytotoxicity *(80)*. For example, chronic high dose ethanol consumption results in high levels of acetaldehyde generation *(81)*. Aldehydes can covalent adduct to proteins through Schiff base reactions and with other cellular components. In addition, CYP2E1 that generates acetaldehyde is loosely coupled to oxidoreductase resulting in the generation of reactive oxygen species. Acetaldehyde can result in exocyclic etheno DNA adducts *(82)*. The resulting oxidant damage and lipid peroxidation can lead to chronic hepatitis. In addition, the marked steatosis that can occur in conjunction with excess alcohol consumption may perturb the insulin/IGF1 signaling pathway of cell survival in the liver *(83)*. Similarly, the one-carbon cycle with eventual folate/choline depletion can contribute to cancer development *(84)*. Ethanol overconsumption in conjunction with HCV increases the risk of cancer development *(85)*. In addition, alcohol abuse in the context of hemochromatosis increases both cirrhosis and HCC risk *(86)*. In part this

may be due to increased oxidant stress in the presence of both increased lipid deposition and increased iron. Low alcohol intake does not appear to be associated with an increased risk of HCC, while higher levels are associated with an increase in risk of both cirrhosis and HCC *(87)*. In some parts of the world, alcohol is made with moldy food staples containing other liver toxins that can compound the problem. Similarly, intake of high levels of iron in conjunction with alcohol can similarly exacerbate the oxidant stress in the liver leading to cirrhosis. Since cirrhosis is associated with more than 60% of HCC in the human *(8)*, this is an important pathway through which ethanol contributes to primary liver cancer development.

Studies in animal models indicate that agents that act through selected nuclear receptors are associated with the ability to regulate cell proliferation/survival, apoptosis, and differentiation can promote tumor development *(18*, *26*, *78)*. Such agents can promote the outgrowth of cells with genetic damage into preneoplastic lesions and hence can under certain circumstances of exposure increase the incidence of hepatic neoplasia in rodents and humans. Tumor-promoting agents are believed to alter the balance between proliferation and apoptosis in initiated cells relative to the normal surrounding cells *(88*, *89)*. Studies with prototypical hepatic tumor-promoting agents including phenobarbital, PPARα agonists, and ethinyl estradiol indicate that a generalized mitosuppression of non-focal hepatocytes is an early and sustained activity of such agents. In addition, reversible alteration of gene expression is associated with tumor promotion. Furthermore, tumor promotion is reversible and exhibits a threshold *(26)*.

3.2.1. Phenobarbital

Phenobarbital and related agents are not genotoxic, yet they can result in the development of cancer in susceptible organisms *(90)*. While selected mouse strains can develop neoplastic lesions following chronic exposure to phenobarbital or related agents, certain rat strains can develop adenomas and rarely adenocarcinomas after chronic exposure. At therapeutic doses, man does not appear susceptible to liver tumor development with chronic phenobarbital administration (c.f. *(91)*). Initiation–promotion studies indicate that phenobarbital has a promoting action *(92)*. Importantly, a dose-dependent promoting activity is observed that exhibits a threshold *(93)*. Interestingly, phenobarbital and related agents can increase the background proliferation rate transiently in the liver *(94)*. Specifically, phenobarbital increases the focal relative to the non-focal hepatic labeling index *(95)*. Importantly, phenobarbital promotes eosinophilic, but not basophilic lesions *(96)*. In addition, a mitosuppression can be observed in the non-focal hepatocytes *(97)*, while the discrete focal hepatocytes have an increased rate of proliferation compared with control hepatocytes or the surrounding normal appearing ones *(98*, *99)*. Phenobarbital increased DNA synthesis and decreased

apoptosis in hepatocytes in vitro *(100, 101)*. Studies with phenobarbital showed that only the promoting dose resulted in changes in gene expression associated with apoptosis suppression and cell proliferation, while dose-dependent changes in selected drug-metabolizing agents were observed *(102)*. It has been suggested that the increased growth rate of the eosinophilic lesions compared with the surround is due to the decreased responsiveness of the altered focal cells to TGFβ family members responsible for apoptosis *(103)*. IGF2R modulates cell proliferation in response to insulin and IGF family members and apoptosis in response to TGFβ. The expression pattern is altered in focal compared with non-focal areas of the liver for IGF2R and TGFβR *(104, 105)*. Phenobarbital can promote those initiated cells with a low level of TGFβR, while increasing ligand expression in surrounding hepatocytes *(103, 105)*. TGFβ is a potent mitoinhibitor of hepatocytes and phenobarbital increases this ligand in non-focal hepatocytes and TGFβ is increased at the protein level during mitosuppression induced by phenobarbital exposure *(103, 106)*.

Previous work has demonstrated that phenobarbital-like compounds cause the increase in gene expression of a number of genes including CYP2B1/2 *(107)* and is transcriptionally regulated *(108)*. The tumor-promoting action of this type of agent is correlated with the induction of CYP2B1 *(109)*; therefore, the mechanism underlying tumor promotion by phenobarbital and related compounds has been associated with the mechanism of CYP2B1 induction. Since a structurally diverse group of compounds act in a similar manner, it has been under consideration as to whether a receptor was responsible for this action. The constitutive androstane receptor (CAR) plays a role in the induction of CYP2B family members *(110)*. Agents that act to alter the metabolism of testosterone derivatives, specifically androstenedione, can alter endogenous activation of the CAR receptor *(111)*. There are two forms of CAR and phenobarbital can displace the ligand from CARβ *(111)*. Agents such as phenobarbital activate the CAR receptor to perturb gene expression *(112–114)*. Studies in knock-out mice indicate that certain genes are expressed or repressed when the CAR receptor is present while a separate set is affected when it is not present *(115–116)*. It is clear that CAR is associated with the gene expression acutely associated with phenobarbital exposure, but how this is associated with tumor promotion is unclear. CAR knock-out mice have been used to confirm that CYP2B expression is dependent on CAR *(114)*. Nonetheless, CAR knock-out mice are resistant to phenobarbital-induced hepatic tumor promotion *(117)*. Interestingly, chronic phenobarbital administration results in DNA hypomethylation that is CAR dependent *(118)*. The mouse strain susceptible to spontaneous and chemical carcinogenesis is sensitive to promotion by phenobarbital, while the resistant strain C57B6l6 is resistant. The tumors arising spontaneously in C3H mice are Ha-ras mutation positive *(119)*, lack

CAR, and are not promoted by phenobarbital *(120)*. These tumors lack CAR, but express β-catenin and are promoted by phenobarbital *(120, 121)*.

Nuclear receptors are frequent targets of drugs and of environmental chemicals. The function of these ligand-activated transcription factor receptors is to regulate endogenous metabolism; hence, homeostasis can be perturbed when their function is modulated. Drugs and environmental chemicals can alter the effects of multiple nuclear receptors due to their broad and overlapping substrate specificity. The interaction of nuclear receptors with coactivators and corepressors provides another level of control of their function within cells. The constitutive androstane receptor (CAR) is a nuclear receptor that regulates the expression of drug-metabolizing enzymes *(112–114)*. CAR is an important regulator of many genes involved in drug metabolism including a number of P450s, phase 2 enzymes, and transporters. Species specificity in response to CAR agonists has been detected although that of phenobarbital (PB) is only 1.5-fold (the human is less sensitive) and human CAR is not sensitive to the same bile acids as mice *(122)*.

3.2.2. Estrogenic Agents

In the human, certain estrogenic formulations can result in adenoma development and rarely in carcinomas. Estrogenic agents can be carcinogenic to rat liver, but tend to inhibit cancer development in the mouse liver. Estrogenic agents are clearly promoting toward the rat liver, but the basis for this action is unknown *(123–128)*. Estrogenic agents can increase cell proliferation in the rat liver and can induce focal proliferation with mitosuppression in the surrounding hepatocytes *(129, 130)*. Examination of altered gene expression during the mitosuppression observed with chronic ethinyl estradiol treatment demonstrated an increase in TGFβ and IGF2R/M6PR without a change in myc or CEBPα levels *(131, 132)*. The increase in TGFβ leads to CKI induction that may lead more directly to the mitoinhibition *(133)*. Similarly, EE exposure induces TGFβ1 expression. Hepatocytes with decreased levels of TGFβR are at a selective growth advantage compared to cells without this characteristic *(105)*. Hepatocytes that survive TGFβ exposure have decreased HNF4α activity, but increased fos, jun, myc, and ras levels *(134)*. Oncogene expression can confer tumor characteristics that TGFβ responsiveness can limit *(135)*; thus, loss of TGFβ responsiveness is permissive to acquisition of the tumor phenotype. In certain, hepatocarcinogenesis protocols administration of tamoxifen results in the regression of a component of the lesions suggesting an estrogen (and estrogen receptor) dependence for those lesions *(136–138)*.

Sustained estrogen receptor activation is known to increase the incidence of liver neoplasms in animals and humans. An increase in adenomas was observed in young women taking an early form of oral contraceptives (with a higher dose and different formulation to the current available forms). Rarely,

HCC was observed in women taking early formulations of estrogens for oral contraceptive purposes *(143)*. Estrogenic agents are effective tumor-promoting agents in the rat liver and their action to initiate cells through catechol estrogen formation *(144)* or induction of aneuploidy *(145)* needs to be assessed at physiological concentrations. For example, certain estrogenic agents can cause a burst of increased proliferation in the rodent liver *(146)*. This transient increase in cell proliferation is associated with stimulation of the estrogen receptor *(126*, *141)*. There is a mitosuppression in the normal appearing hepatocytes, while the focal, putatively, preneoplastic hepatocytes have a sustained increase in proliferation *(131*, *141*, *146)*. Although the incidence of HCC in humans following chronic (greater than 5 years) estrogen exposure is low, the incidence is definable and permits one to anchor the incidence in rats where a clear carcinogenic response to high dose, potent carcinogens is observed under defined exposure conditions. This observation permits more accurate risk assessment from animal hazard identification studies. Extrapolation of potential for risk across species could be performed using the low incidence human tumor data as an anchor for the calculations.

Estrogenic agents have a carcinogenic potential at several sites including the mammalian liver *(144)*. Estrogenic agents are known liver tumor-promoting agents in the rat *(124*, *125*, *137)* and in the human *(145)*. There is an apparent threshold for promoting action *(146–148)*. The mechanism of tumor promotion is not known although an increase in focal proliferation and a decrease in focal apoptosis have contributing roles. Although tamoxifen has an estrogenic action in the liver that may contribute to its promoting action, the phenotypes of the liver lesions that arise with mestranol and tamoxifen treatment differ *(149)*. In addition, tamoxifen can inhibit the development of mestranol-promoted lesions indicating a divergent mechanism of action *(126*, *137)*. The mechanism of estrogenic/antiestrogenic action for tamoxifen is only incompletely understood. While this action may in part be due to an interaction with the estrogen receptor, other factors may also be involved. For example, antiestrogens bind to sites other than the estrogen receptor including covalent binding to P450s *(150)*, tubulin *(151)*, and other interactions with "antiestrogenic binding sites" *(152)*. In addition, antiestrogens inhibit protein kinase C and calmodulin activity *(153)*. In addition, antiestrogens alter the production of several peptide growth factors including TGFα *(154)*, TGFβ *(155)*, and IGF1 *(156)*, and affect some calcium-dependent processes *(157)*. Estrogenic and antiestrogenic agents additionally alter cholesterol metabolism *(152)*. Tamoxifen appears to promote the diploid hepatocyte population *(158)*, similar to ethinyl estradiol *(159)*. The triphenylethylene antiestrogens have differential effects on the hepatic proliferative rate in the rat *(160*, *161)*. In the liver itself, triphenylethylene antiestrogens have an estrogenic action; however,

these drugs are mixed agonist/antagonists in a species, strain, tissue, gene, and hormone status basis.

Mestranol is a synthetic steroidal estrogen that is metabolized *(162)* to the potent rat liver tumor-promoting agent, ethinyl estradiol *(154)*. Mestranol use in oral contraceptives was associated with an increased incidence of hepatic adenomas and a few hepatocellular carcinomas in young women *(144, 163–165)*. Studies in rats indicate that mestranol and its active metabolite ethinyl estradiol promotes the development of previously initiated liver cells through induction of elevated cell proliferation levels. Mestranol does not have a marked effect on P450 profiles in the liver *(166)*, but it can cause cholestasis *(167)* and clearly enhances liver growth *(166)*. Chronic administration of ethinyl estradiol results in mitosuppression of liver cells with selection of resistant hepatocytes for outgrowth *(129, 143)* and this in combination with its ability to increase cell proliferation *(126, 168)* is believed to be responsible for its tumor-promoting properties *(123, 125, 126, 129, 143, 148, 169, 170)*. Tumor promotion by ethinyl estradiol is effected through the estrogen receptor, since it can be inhibited by tamoxifen *(137, 138)*. At low doses and for short durations of administration, ethinyl estradiol can increase hepatic hypertrophy and a transient increase in cell proliferation *(126, 168)*, while with chronic administration a mitoinhibition is observed *(126, 129)*.

3.2.3. PPAR Agonists

The peroxisome proliferators-activated receptors (PPARs) are members of the steroid/retinoid receptor superfamily. Three mammalian nuclear receptors of the PPAR class have been isolated including PPAR alpha, delta, and gamma *(171)*. The PPARα receptor is a ligand-activated nuclear transcription factor that is responsible for the regulation of lipid catabolism *(172)*. The PPARα receptor and the retinoid X receptor nuclear receptor (RXR) can heterodimerize and bind to peroxisome proliferator response elements (PPRE) to alter the transcription of genes including those that are involved in lipid metabolism *(173–175)*. Peroxisome proliferators include structurally diverse chemicals that can activate the PPARα receptor including industrial chemicals, plasticizers, herbicides, and some lipid-lowering drugs *(175–177)*. Agonists of PPARα induce peroxisome proliferation *(177, 178)*, hepatomegaly *(177, 179)*, cell proliferation *(177, 180, 181)*, and liver neoplasms in rodents *(175, 181, 182)*. Although numerous theories exist regarding the mechanism of hepatocarcinogenesis in the rodent following chronic exposure to PPARα agonists, the mechanism is not fully understood. In general, PPARα agonists are not genotoxic and demonstrate a promoting activity *(183)*. Similar to other receptor-mediated, non-genotoxic rodent carcinogens, PPARα agonists, including WY14, 643, methylclofenapate, nafenopin, and clofibric acid increase the TGFβ1 ligand, while these agents

excluding clofibric acid increase expression of the IGFII/Man6P receptor *(184)*. Sustained PPARα receptor activation is required for induction of liver tumors, since PPARα knock-out mice do not develop hepatic neoplasms even after a 1-year exposure to PPARα agonists *(185)*. Similarly, peroxisome proliferation and gene expression regulated by PPARα are not altered by exposure to PPARα agonists in the knock-out mice *(185)*. The lack of carcinogenic action in the human relative to the rodent has been explored with human PPARα receptor knock-in mice *(186)*. Although the precise mechanism of the hepatocarcinogenesis of PPARα agonists in rodents is not fully understood, it appears to be dependent upon PPARα receptor activation *(187–189)*. Thus, PPARα agonists are non-genotoxic carcinogens that function through receptor activation *(190)* and appear to be carcinogenic in the rodent, but not in primates.

3.2.4. AhR Agonists

The aryl hydrocarbon receptor (AhR) is structurally distinct from the nuclear receptors and contains a bHLH-PAS domain *(191–193)*. The ligand-bound receptor interacts with arnt and this dimerization partner regulates the expression of specific genes. The ligand-binding domain of AhR is within the PAS domain. The PAS domain of AhR binds ligand, binds to a repressor (probably hsp90) and has some of the interaction function with arnt. The function of excess AhR ligand may be to block the function at the other sites of arnt binding. The low-affinity allele of AhR found in some mouse strains is similar to that observed in humans *(194–196)*. In addition, the transactivation domain part of AhR is highly divergent with only a 60% identity between rat and human *(196)*. This suggests that human gene expression in response to an AhR ligand will differ qualitatively as well as in magnitude from that in rats and mice containing the high-affinity AhR allele.

TCDD and related agents can induce a range of toxicities that may be mediated by AhR *(191)*. Dioxin lacks any genotoxic activity, yet increases the incidence of hepatic neoplasms in rats *(197)*. Dioxin can cause marked cytotoxicity at higher doses and this may contribute to its tumor-promoting activity. Activation of arylhydrocarbon receptor (AhR) by 2,3,7,8-tetrachlorodibenzoparadioxin (TCDD) and related compounds of the furan and PCB classes results in alterations in gene expression including an induction of CYP1A1 *(198)*. Although the role of CYP1A1, if any, in tumor promotion is unclear, CYP1A1 expression is a useful marker for ascertaining exposure to this class of compounds. Over 100 genes may be regulated by AhR activation *(199)*. Genetic differences between mouse strains have been used to demonstrate that TCDD-mediated liver tumor promotion is AhR dependent *(200)*. Transgenic mice overexpressing a constitutively active AhR are more sensitive to diethylnitrosamine initiation resulting in a higher yield of preneoplastic lesions than the genetically matched control

animals *(201)*. Knock-out animals have been generated *(202–204)*. The gene expression patterns *(205)* and toxicity *(206)* have been examined after acute but not chronic administration of TCDD to the knock-out animals. The genetic background of the animal is important for its potential to develop neoplasms in response to TCDD administration. Since a selection for neoplastic clones resistant to the toxic insult that permits their outgrowth occurs, Ha-ras-mutated hepatocytes might be resistant to Ahr-dependent toxicity. Liver tumors from TCDD-treated mice have a high incidence of Ha-ras mutations *(207)* suggesting that the C3H background would be exquisitely sensitive to TCDD-induced tumor promotion *(121)*.

Initiation–promotion studies in the rat *(208*, *209)* indicate that there is a threshold for the promoting action of TCDD and related compounds. A variety of studies indicate that TCDD causes a generalized mitosuppression in the liver *(210*, *211)*. However, an increased cell turnover in focal lesions was noted relative to the surrounding liver *(212)*. The initiated cell population is resistant to apoptosis *(213)*. Interestingly, the AhR null hepatocytes both secrete TGFβ ligands and are quite sensitive to the apoptosis induced by TGFβ *(214)*, indicating that AhR deficiency leads to increased TGFβ ligand production wherein selection for resistance to its apoptotic effects would permit promotion. Perhaps, TGFβR or processing of TGFβ through IGF2R would confer selective growth advantage to AhR–/– mouse hepatocytes that secrete TGFβ ligands. The AhR null mice have been used to demonstrate that the gene induction profile associated with AhR activation are altered *(205)* and the acute toxicities associated with AhR activation are diminished *(206)*. For example, CAR is increased by AhR activation *(215)*, while growth hormone receptor and janus kinase 2 are decreased *(216)*. Future studies should address the question of carcinogenicity in mice with AhR overexpressing and null alleles on different mouse strain backgrounds. In the human, exposure to TCDD has been associated, but not causally linked to an increased cancer risk *(217*, *218)*. In part, the human AhR receptor is less sensitive to activation by AhR ligands *(196)* and in part, the exposure level in humans has been below that required to cause sustained tumor promotion *(219)*. Other agents in the class including certain polychlorinated biphenyls and the tetrachlorofurans may act in part through an AhR-dependent mechanism. Each agent has a unique contribution of AhR, CAR, and ER-dependent activity as well as other actions including cytotoxicity that may contribute to its carcinogenicity in rodents and provide a potential risk to the human. Certain exposures to mixtures of PCBs and furans have been associated with an increased risk of human liver disease and cirrhosis *(220)*, but a causal link has not been made to cancer. Even in worker populations, the low incidence and lack of consistent dose trend prohibits the conclusion of causality *(221)*. The risks at high dose exposure differ from the risks posed by ambient exposures and should not be oversimplified.

3.2.5. Ethionine

Ethionine, an antimetabolite of the amino acid methionine, when administered in the diet for extended periods can result in the development of liver cancer in rats *(29)*. This was the first example of direct interference with the metabolism of a normal metabolic constituent, resulting in the development of cancer. Ethionine induces marked steatosis that progresses to NASH, cirrhosis, and HCC *(30, 222)*. Its ability to disturb one-carbon pools (rats are ten times more sensitive than humans to choline deficiency), folate metabolism, and to induce steatosis is similar to alcohol-induced changes that progress to cirrhosis and ultimately to HCC. This compound interferes with methylation causing hypomethylation upon chronic administration *(223)*. This agent is not used in the human.

4. PATHOGENESIS OF HCC

The pathogenesis of human HCC has been examined extensively *(6–8, 81)*. Generally, the neoplasms are detected at late stage when many concurrent genetic changes are apparent. Tracing the earliest genetic changes in clinical samples has been limited. Studies using CGH arrays and gene expression analysis indicate that multiple pathways and multiple mechanisms lead to HCC development and progression due in part to different etiologies and time during pathogenesis of clinical detection. Primary liver cancer associated with cirrhosis evolves from precancerous lesions. Dysplastic nodules have variable degrees of atypia and can exhibit a focus or nodule in nodule appearance that can range from normal appearing to neoplastic in appearance. The formation of dysplastic nodules is not required for HCC development. Large cell dysplasia appears to be a response to injury and is not strictly a preneoplastic lesion although it is associated with an increased risk of HCC in a cirrhosis background of more than 3-fold *(6)*. On the other hand, small cell dysplasia seems more characteristic of preneoplastic change with greater than a 6-fold risk *(6)*. These small cell dysplastic cells are more diploid and less differentiated in character than the large cell dysplasias.

4.1. Rodent Models of Hepatocarcinogenesis

Examination of the epidemiology of liver cancer in humans indicates that both genetic and environmental factors are involved in the etiology and evolution of this disease. Studies in rodents can provide insight into the various factors involved in liver carcinogenesis. Early studies on rodents exposed to carcinogens indicated that male rodents are more likely to develop liver tumors *(224, 225)*. Rats, although relatively resistant to the spontaneous induction of liver neoplasms, will develop hepatic tumors later in life with

a sex-bias in incidence that differs between strain and study *(226)*. This compilation of strain background effects on spontaneous liver tumors in rats suggests that females have a slightly higher rate in Charles River CD, Osborne–Mendel, and Fischer rats and the incidence in males being marginally greater in the Wistar strain. Hepatic tumors can be readily induced in the rat by a variety of carcinogenic agents, with the male generally more sensitive than the female. The cancer bioassay is performed in two species of rodent, the rat and mouse. The sex specificity of liver tumor induction is, however, carcinogen specific due in large part to the sex dependence of the metabolic pathways.

4.2. Rat Models

The rat liver has been used extensively as a model of the carcinogenic process *(5, 17)*. Three basic protocols with numerous variations have been described including resistant hepatocyte model, neonatal rat model, and the partial hepatectomy model. These models couple carcinogen administration with a period of rapid cell proliferation due to the intrinsic growth of the tissue in the neonate, the wave of proliferation that occurs following surgical resection, or the extensive necrosis induced by excessive carcinogen administration. These studies can be used to examine very early changes in the pathogenesis of preneoplasia in the rat liver. The initiation–promotion–progression (IPI) model *(227)*, the Solt–Farber model *(228)*, and transgenic *(229)* rat models can be used to analyze later focal hepatic lesions, adenomas, and carcinomas. The utility of the rodent as a model lies in the ability to assess the changes associated with early premalignant changes that would not be detected in clinical samples that present late in the progression process. In addition, rodents can be used to model gene–environment interactions in a controlled manner. Thus, the early premalignant changes, as well as the initial stages and pathways in progression of primary liver cancer are tractable in rodent models, while human cases are more amenable to analysis of later progression.

The rat has been used extensively as a model to examine the process of liver cancer development and to ascertain which compounds can influence cancer development in the liver. Studies by Bannasch *(230)* indicate that two pathways that evolve toward HCC in the rat are thyroid mimetic and insulin mimetic (insulin-signaling pathway) with resulting glycogen accumulation phenotype. With progression, a shift from anabolic to catabolic glucose utilization occurs in the insulin-dependent signaling pathway. Similarly in humans, diabetes mellitus predisposes to HCC development as an independent risk factor *(15)*. This effect is observed in livers of rats treated with phenobarbital and related types of agents that promote eosinophilic lesions, while a thyroid-like effect is observed for the basophilic lesions that

arise with PPARα agonist administration. Although PGST has been used as a marker of putatively preneoplastic lesions in the rat and is increased in expression in single cells following carcinogen exposure, in focal lesions with promotion, and in some neoplastic nodules and neoplasms, a deficiency of glucose-6-phosphatase expression may be more representative of hepatic lesions that will progress to neoplasia.

Analysis of the gene expression changes across the carcinogenesis process and especially in preneoplastic lesions or following carcinogen exposure can illuminate the processes impacted by carcinogens. Recently, gene expression analysis has been applied to gain a clearer understanding of the changes that accompany liver cancer development in the rat. Many of these studies have been performed using variations on the Solt–Farber selection model for rat liver cancer induction *(228)*. Preneoplastic lesions have a higher level of expression of genes that are anti-apoptotic (p53, NK-kB, and Bcl-2 pathways) and pro-proliferation *(231)*. Proliferation gene changes are also common in liver tumors, while apoptosis was decreased *(232*, *233)*. Early nodules demonstrate a decrease in both growth hormone receptor and growth hormone binding proteins *(234)*. Specifically, IGF2 is expressed during liver cancer development, while IGF1 is decreased during liver cancer development *(235)*. These more fetal-like gene expression patterns are observed during early tumor development *(236)*. The increased expression of TGFα and HGF and their respective receptors, EGFR and met, observed in early nodules is lost with neoplastic progression *(237)*. Gene expression analysis demonstrates many genes in common between neoplastic nodules and HCC with only a few genes uniquely observed in HCC *(231*, *237)*.

4.2.1. Multistage Nature of Cancer Development

Molecular analysis of the pathogenesis of the natural history of liver cancer induction and progression has been extensively examined in the rodent. In the rat, single hepatocytes aberrantly expressing glutathione-*S*-transferase P (GSTP) can be observed within 2 days of carcinogen exposure *(238–243)*. Under many conditions, GST expression has been suggested to represent a population of initiated hepatocytes in the rat liver *(240*, *241*, *243)*. This is true for several types of genotoxic carcinogens including diethylnitrosamine *(238*, *243)*, an alkylating agent, aflatoxin B1 *(238)* that results in the formation of bulky DNA adducts, and choline-deficient diet that results in depletion of methyl pools *(242)*. Single GSTP-expressing hepatocytes are found in a dose-dependent manner following carcinogen administration *(238)*. Some subset of these cells will grow into colonies of hepatocytes also expressing GSTP. These findings suggest that the single GSTP-expressing cells are precursors of those that form colonies and by definition of some of those that will progress into hepatic neoplastic nodules and HCC. Single hepatocytes expressing GST have the characteristics associated with

initiated liver cells; namely, dose-dependent induction with carcinogen administration, rapid appearance after carcinogen treatment, enhanced intrinsic proliferation compared with surrounding apparently normal hepatocytes, and response to the selective growth pressure exerted by a promoting agent *(238)*. Expression of genes at the single cell level has been inadequately characterized, but GSTP and GGT are increased in certain hepatocytes following carcinogen administration.

4.2.2. Promotion

The promotion stage of cancer development has been operationally defined as the clonal expansion of the initiated cell population. The growth kinetics of GST-expressing hepatocytes can be followed over time through the analysis of the size and volume fraction of the liver occupied by GST-expressing hepatocytes *(238)*. The hepatocytes within AHF during promotion are primarily diploid *(244, 245)* and additionally lack demonstrable karyotypic changes *(245)*. Promoting agents stimulate the growth of the focal hepatocytes in a reversible manner and this can be determined by assessment of the size of the observed (GST expressing) hepatic lesions and by determination of focal increase in the expression of cell proliferation markers *(246)*. The net growth rate of GST-expressing hepatocyte colonies can be determined from the volume fraction occupied by such lesions as a function of time. The net growth rate thus reflects the balance between the birth and death rate within this population in relation to that observed in the surrounding apparently normal cells. While many of the GSTP-expressing lesions will regress, the nodules that concurrently express GSTP and gamma-glutamyltranspeptidase (GGT) appear to be the ones that progress. The loss of expression of glucose-6-phosphatase has also been associated with progression, but it is unclear whether this is through a different mechanism than for GSTP-expressing lesions. Gene expression has been examined in these early putatively preneoplastic lesions that precede nodule in nodule of HCC.

4.2.3. Progression

The stage of progression encompasses the spectrum of changes that occur in the conversion of preneoplastic cells into malignant neoplasia *(247)*. There is not as yet a validated method for the quantitation of hepatocytes in the stage of progression. This stage is characterized by an evolving karyotypic instability and aneuploidy indicating the necessity of understanding alternative pathways in progression of liver neoplasia. Morphologically, the focus in nodule configuration is the earliest endpoint for detection of progression in the liver *(227, 248, 249)*.

4.3. Mouse Models

Certain mouse strains are more susceptible to spontaneous *(224)* and chemically induced *(250)* hepatic tumors than other strains. An upregulation of c-jun may mark single altered cells in the mouse liver *(251)* analogous to the increased GSTP expression in the rat. The focal areas of change can be detected in frozen sections by the loss of expression of glucose-6-phosphatase. Alternatively, H&E stained sections demonstrate the presence of two distinct lesion types (A and B). Discussions by Schwartz indicate that one class contains Ha-ras mutations, while the other class contains β-catenin mutations. The C57Bl/6 (resistant) and the C3H (sensitive) strains differ in their susceptibility to spontaneous and chemically induced liver cancer development *(252)*. The hepatocarcinogenesis susceptibility allele (Hcs) is autosomal and is inherited in a semi-dominant manner with the F1 between the sensitive and resistant strain demonstrating an intermediate phenotype. This phenotype is believed to be cell autonomous factor *(253)*. In a study performed by Drinkwater et al. *(254)*, BXH (RI strains developed from a cross between C57Bl/6 (B) and C3H (H) mice were subjected to neonatal ENU administration. BXH strains 6, 14, and 10 were resistant, while BXH strains 8, 9, 7, and 3 were sensitive to ENU-induced increases in liver tumor multiplicity. A number of susceptibility gene loci have been described genetically for mouse liver cancer development. These cancer modifier loci have been mapped to specific chromosomal locations based on the Mendelian inheritance patterns in inbred mouse strains that are sensitive and resistant to cancer development *(255)*. Strain differences in sensitivity to liver cancer development were described by Andervont *(255a)* indicating a genetic component to the spontaneous development of liver cancer in mice. A few of these genes have been identified by positional cloning approaches. In addition, human homologues of cancer sensitivity and resistance alleles have been proposed. The C3H strain is susceptible to spontaneous and carcinogen-induced liver cancer development, while the C57/Bl6 mouse is by comparison resistant. The hepatocarcinogenesis sensitivity (HCS) and resistance (HRS) alleles have been defined for the mouse. A hepatic susceptibility locus on mouse chromosome 1 accounts for 85% of the variance between these two mouse strains *(252*, *256)*. Studies with other mouse strains and other carcinogens have also been performed *(157)*.

The National Toxicology Program assesses cancer risk in the C3B6 F1 mouse that carries the dominant susceptibility allele for liver cancer development. The most common experimental cancer assessment tool is the neonatal mouse model *(257)* as first described by Vesselinovitch *(258)*. Numerous models of human liver diseases exist. Many of these are developed as a complicated toxin or carcinogen regimen *(17)*. In addition, genetically modified mice have been made against signaling pathway members

believed important in liver cancer development *(229)*. These rarely are a complete recapitulation of the human disease, but are nonetheless useful for modeling one component of the disease. The challenge is to couple etiologic agents, with pathway perturbations and disease models to unravel components of the pathogenesis of human primary liver cancer *(17*, *229*, *259)*.

Analysis of early and progressive lesions that arise in the mouse, rat, and human will provide a mechanism by which to develop models of human liver cancer development, pathogenesis, and progression.

5. ETIOLOGY IN THE HUMAN

Patients at risk for HCC include those with chronic hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infection *(260)*, certain metabolic liver diseases, such as hereditary hemochromatosis *(261)*, Wilson's disease, alpha-1 antitrypsin deficiency, and porphyria cutanea tarda *(7*, *8)*. Individuals with cirrhosis are at risk of HCC *(7*, *262)*. Heavy alcohol consumption is also a common major risk factor for developing HCC *(7*, *8*, *83*, *85*, *262)*. Other predisposing factors include gender (males are times more likely to develop HCC than females), smoking, and diabetes *(262)*. Environmental influences, including carcinogen exposure and viral hepatitis prevalence, are believed to contribute to its distinct geographical distribution pattern *(8)*. Specifically, chronic infection with HBV and exposure to aflatoxin in the diet contribute to high-risk levels of HCC *(263)*. Thus, primary liver cancer is a product of environmental exposures with genetic consequences. In the United States, the largest cross-sectional study of HCC identified infection with HCV and/or HBV as the most common risk factor for HCC (47% HCV, 15% HBV, 5% both). Approximately, 33% of primary liver cancer in the United States are not associated with HBV or HCV *(8)*. The incidence of HCC is increasing in the United States primarily due to an increase in hepatitis C virus infection *(8)*.

5.1. Cirrhosis

Individuals with cirrhosis, regardless of its etiology are at risk for HCC *(7*, *262)*. Fibrosis of the liver can result as a response to liver injury or as a component of selected genetic diseases *(264*, *265)*. Cirrhosis is the end stage of fibrotic disease. Cirrhosis of the liver can occur during the progression of alcoholic hepatitis, non-alcoholic steatohepatitis (NASH), viral hepatitis, and cholestatic liver diseases *(266)*. Viral hepatitis (HBV and HCV) and alcohol are the primary causal factors in liver cirrhosis, while NASH, certain genetic diseases (e.g., hemochromatosis), and immune-mediated

damage provide other contributing factors *(7, 8)*. There is an increased risk of primary liver cancer in individuals with hepatitis C-associated cirrhosis and diabetes mellitus *(267)*. In some conditions, cirrhosis can progress to hepatocellular carcinoma.

5.2. Non-alcoholic Steatohepatitis (NASH)

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of elevated serum enzymes indicative of liver injury and may be due to many etiologies *(268–270)*. An independent diagnostic test or disease marker is not available for NAFLD. The NAFLD disease continuum, which has a worldwide prevalence of 20%, is defined to exclude viral hepatitis, autoimmune diseases, metabolic changes due to hemochromatosis, alpha-1 antitrypsin, and ceruloplasmin changes, and alcoholic liver disease despite the similarities of disease presentation. Steatosis appears to be a benign condition, but steatohepatitis is progressive *(268–270)*. Essentially all morbidly obese individuals have NAFLD and approximately 25–50% exhibit steatohepatitis. For non-alcoholic steatohepatitis (NASH) patients (prevalence of 1–5% in the general population) approximately 20% will progress to cirrhosis, with a small percentage of these progressing to hepatocellular carcinoma. Approximately 10% of individuals with NASH will die of liver-related diseases *(269)*. NASH is common in type 2 diabetes and has a prevalence of 60% *(269–271)*. Morbid obesity is another risk factor for NASH. Approximately, 2–3% of lean individuals exhibit NASH, while 15–20% of obese individuals have steatohepatitis at non-liver initiated autopsies. Individuals that have insulin resistance are susceptible to the development of steatosis (fatty liver) and its progression to non-alcoholic steatohepatitis (NASH). In some individuals, steatohepatitis can progress to cirrhosis and in a limited number of cases can progress to primary liver cancer *(271)*. Recently animal models of NAFLD and NASH have been developed, but these do not completely recapitulate the pathogenesis of the related diseases and do not progress to cirrhosis or HCC without additional provocation *(272)*. Current trends suggest that the NAFLD continuum is not as benign as once thought and that progression to NASH, cirrhosis, and potentially HCC can occur depending on the interaction of genetic, environmental factors, and underlying disease including diabetes, HFE, among others *(273–276)*.

5.3. Viral Hepatitis

Chronic infection with HBV or HCV is the predominant risk factor for development of HCC, accounting for up to 80% of liver cancer cases in geographic regions of high incidence of the disease *(7, 8, 277)*. Although much of the HCC incidence is attributable to chronic HBV infection, only a

low percentage of individuals that are infected with HBV go on to develop progressive liver disease even though 80% or more develop chronic infection. Approximately one third of individuals with chronic infection will develop cirrhosis and HCC develops in less than 5% of those that develop cirrhosis *(278)*. Carriers of HBV have 100-fold risk of developing HBV *(277)* that has been suggested to be closer to 5–15-fold in case–control studies with a lifetime risk of 10–25%. The annual incidence in HBV carriers is less than 1% *(279)*. It increases to greater than 1% in those with hepatitis and to 2–3% in those with cirrhosis. Although rates of infection with the viruses are similar in men and women, there is some evidence that progression of the disease is more likely to occur in men *(7)*. Among chronic carriers of hepatitis B surface antigen (HBsAg) in Taiwan, the ratio of men to women was 1.2 for asymptomatic individuals, but there were six times as many men as women among patients with chronic liver disease *(278)* in concert with the greater prevalence of chronic hepatitis and cirrhosis in men *(278)*. A prospective study of liver cancer development among men in Taiwan has indicated a relationship between serum testosterone levels and risk for HCC *(278–281)*. Men, whose testosterone levels were in the highest tertile (>5.7 ng/ml), had a relative risk of 2 for development of HCC when compared with men having lower testosterone levels *(280)*. When other risk factors, including HBsAg carrier status, anti-HCV positivity, and alcohol consumption, were taken into account, the relative risk for men with high testosterone levels was 4 *(279*, *281)*. However, this difference may have been due to a higher proportion of HBsAg carriers among the liver cancer cases. In developed countries, HCV infection is a more prevalent risk factor for HCC. HCV infection results in a 15-fold increase in risk of HCC compared with uninfected individuals. Approximately, 90% of HCV carriers develop hepatitis, while 20% of HCV carriers develop cirrhosis. Cirrhotic HCV patients develop HCC at a rate of 1–4% per year *(7*, *8*, *279*, *281)*. The high rate of cirrhosis development results in a risk of HCC over the lifetime of 1–3%. The risk of HCC is further increased in HCV carriers for alcohol excess and HFE carriers *(279*, *281)*.

5.4. Aflatoxin and Other Dietary Carcinogens

A number of dietary factors have been associated with HCC risk including exposure to aflatoxin (a fungal product of *Aspergillus flavus* and related species). The risk of HCC is exposure (dose and duration) dependent *(26*, *282)*. The risk is heightened in those with HBV *(283)*. This toxic substance is produced by certain strains of the mold *Aspergillus flavus*. Aflatoxin B_1 is one of the most potent hepatocarcinogenic agent known and has produced neoplasms in rodents and primates *(26)*. This agent is a potential contaminant of many farm products (the common food staples, grain, and peanuts)

that are stored under warm and humid conditions for some time. Aflatoxin B_1 and related compounds may cause some of the toxic hepatitis and hepatic neoplasia seen in various parts of Africa and the Far East *(284)*. Thus, an important environmental and experimental hepatocarcinogenic agent is aflatoxin B_1. Other products of molds and fungi are potentially carcinogenic in humans and animals including fumonisins *(285)*. Other fungal *(286*, *287)* and microbial products *(288)* may similarly be associated with HCC risk. Certain alkaloids are cytotoxic to the liver and may be associated with an increased risk of liver cancer. A number of plants, some of which are edible, also contain chemical carcinogenic agents whose structures have been elucidated *(289)*. These include the pyrrolizidine alkaloids which are found in comfrey and riddelline *(290)*. The use of Senecio, Crotalaria, Heliotropium, and Symphytum species can result in veno-occlusive disorder. Acute toxicity can occur with high dose exposure, but lower doses and longer durations of treatment can result in chronic disease. While these agents are used as teas and herbal remedies, they have been associated with acute toxicity and when there is a genotoxic metabolite in addition to cytotoxicity the combination of DNA adduct formation and cell proliferation permits mutation induction and fixation. Similarly, a low intake of retinoids, selenium, vitamin E and other antioxidants may also be associated with an increased risk when combined with other risk factors *(291–295)*.

5.5. Alcohol and Tobacco

Alcohol abuse has been associated with HCC development that occurs in a background of hepatitis and cirrhosis *(81*, *262)*. Alcohol abuse can potentiate HCV and HBV to increase the incidence of HCC *(87)*. This incidence is markedly increased in individuals with high AFP levels, high cell proliferation index, and in uncompensated patients with atypical macroregenerative nodules. In those with compensated liver fibrosis, the risk of HCC is 3% *(87*, *296*, *297)*. Both case–control and prospective studies have indicated that excessive alcohol consumption increases the risk of liver cancer development by up to 3-fold, a result likely due to the induction of liver cirrhosis *(296*, *298*, *299)*. Liver cirrhosis due to excessive alcohol intake is an important risk factor in countries with a low incidence of HCC. Since chronic alcohol abuse is more prevalent among men than women, this risk factor may also contribute to the higher incidence of HCC in men than women *(300)*. Alcohol abuse may be an independent risk factor for HCC in areas of endemic HBV or HCV infection with an attributable risk of approximately 20% in one study *(299)*. Alternatively, associations between gender and lifestyle-associated risk factors, including smoking and alcohol consumption, have been suggested as potential determinants of the sex difference in HCC risk resulting in a male bias in the prevalence of this disease. There

is a positive impact of cigarette smoking on HCC risk *(301–307)*. However, higher rates of HCC are observed in heavy smokers when all other risk factors were taken into account *(307)*. Thus, the lifestyle factors of smoking and alcohol intake contribute to the induction and progression of HCC in a dose dependent and synergistic manner in both high- and low-risk geographical areas *(304, 305)*. Alcohol abuse can increase the risk of HCC in hepatitis virus carriers by at least 2-fold *(87)*.

5.6. Steroids

The factors underlying the sex difference in human risk of developing liver cancer have not been determined. However, the geographical and ethnic diversity in the populations at risk indicate that sex hormone-related factors may underlie the higher incidence of liver cancer development in men. Similarly elevated levels of testosterone result in an increased incidence of hepatic adenomas *(308)*. In men taking anabolic steroids, an increased incidence of liver adenomas has also been observed *(309–311)* and these lesions may or may not regress upon cessation of androgen therapy *(312–313)*. Oxymetholone, methyltestosterone, and danazol administration were associated with hepatic neoplasms in certain cases. HCC was associated with oxymetholone and methyltestosterone in some patients, while adenomas were associated with danazol exposure *(311)*. These studies support the potential for elevated testosterone levels to contribute to the development of hepatocellular carcinoma development *(263, 278)*. Significant associations have been observed between polymorphisms in three hormone-related genes and HCC. These include androgen receptor, 5-alpha reductase, and cytochrome P450 17 alpha *(263)*.

Exposure to either anabolic steroids or certain oral contraceptive formulations has been associated with the increased incidence of hepatic adenomas and in rare instances with HCC development in humans. The earliest report of an association between liver cancer induction and exposure to exogenous sex hormones described seven cases of benign hepatomas in young women with a history of oral contraceptive use *(314)*. Women of child-bearing age appear to be sensitive to the induction of benign hepatic adenomas and the induction of these liver tumors is enhanced by exposure to oral contraceptives. These tumors respond to hormonal manipulations such that they regress upon cessation of hormonal administration *(145)* and grow or progress upon continued administration of these agents. While a dose (estrogenic potency) and duration effect is seen for oral contraceptive use and adenoma development, the association with carcinoma induction is very low and only detectable with greater than 8 years of exposure *(315)*. Several investigators reported that the relative risk for adenoma development increased sharply beyond 5 years of oral contraceptive

use *(316, 317)*. While formulations containing mestranol and ethinyl estradiol have led to equivalent risks, the incidence of liver cancer among women using high potency oral contraceptives was significantly greater than that for users of low-potency formulations. Oral contraceptive use has also resulted in an increased risk for malignant liver cancer *(318)*. Case–control studies in the United States, Britain, and Italy demonstrated a 5-fold increased risk for hepatocellular carcinoma among women with more than 5 years use of oral contraceptives relative to women with exposures of shorter duration *(315, 318–320)*. In contrast, estrogen replacement therapy does not increase the risk for hepatocellular carcinomas *(315)*. Thus, excess exposure to hormonally active agents can increase the risk of HCC.

5.7. Genetic Disorders

A number of metabolic diseases have been associated with an increased risk of HCC *(7, 8)*. These include hemochromatosis, tyrosinemia, citrullinemia, porphyrias, and alpha-1 antitrypsin. Individuals with cirrhosis and genetic hemochromatosis have a markedly increased rate and shortened time until HCC development that is exacerbated by viral infection and alcohol abuse *(263, 278)*. Other metabolic diseases can increase the risk of HCC but to a lesser degree. These include Wilson's disease, fructose intolerance, and type I and III glycogen storage disease. Thus, the variety of the underlying disease base that contributes to HCC demonstrates the multifactorial risk profile for primary liver cancer development.

5.7.1. Metal Overload Disorders

Iron overload *(260, 321)* has been associated with hepatic fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Hereditary disturbances in iron uptake *(322–324)* and metabolism result in one form of iron overload and dietary ingestion excess *(325)* a second. A variety of iron overload conditions have been associated with HCC even in the absence of cirrhosis including sideroblastic anemia and thalassemia *(321, 326)*. In certain areas of sub-Saharan Africa, the natives ingest drinks with concentrated iron. These individuals have an increased incidence of both cirrhosis and HCC *(325)*. Porphyrias occur due to defects in the heme biosynthetic pathway. Both acute intermittent porphyria and porphyria cutanea tarda have been associated with an increased risk of HCC *(324)*. The mechanism is unknown, but the presence of free iron in the tissue may be a contributory factor. In combination with HBV infection, HCV infection, alcohol cirrhosis, iron overload induced an increase in lipid peroxidation and the rate of progression to steatohepatitis, cirrhosis, and HCC *(86, 262)*. Underlying liver

disease including cholestasis, steatosis, and cirrhosis can impact the degree and latency to disease onset and progression with iron overload syndromes.

Hereditary hemochromatosis was first described as a hereditary disease associated with HLA linkage and a form of pigment-associated cirrhosis typically associated with diabetes. A prevalent gene mutation *(323)* was found to underlie hereditary hemochromatosis (HFE) and a knock-out mouse *(327)*. Although several genetic factors can be involved in iron overload, the most common is in HFE (85–90%). Although several polymorphisms exist, the most prevalent is C282Y (85–100% attribution to HFE). The prevalence is 1 in 250 with an allelic frequency of 5%. The second polymorphism allele that is common in HFE is H63D. Carriers of this allele comprise 15–20% of the American population, but the consequence of this allele is not known *(323)*. The HFE is an MHC class 1 molecule that is associated with β2 microglobulin (B2M) and the major polymorphism C282Y prohibits this interaction. Studies in a B2M knock-out mouse demonstrate an iron overload syndrome *(328)*. In the HFE knock-out mouse, periportal iron deposition occurs in conjunction with elevated transferrin saturation *(327)*. Interestingly, HFE and B2M which are in a complex with transferrin receptor result in an increase in intestinal iron absorption. HFE mutation carriers cannot facilitate iron uptake by transferrin receptor *(329*, *330)*. Transferrin receptor Ser142 alleles are increased in liver cancer cases and in addition, TfR expression is increased in hepatic preneoplasia and in HCC *(330)*. The odds ratio for C282Y allele carriers with TFR142Ser alleles for HCC is 17.2, while it is 62.8 in those with cirrhosis for HCC development demonstrating the contribution of TfR to risk of HCC *(330)*.

The long-term consequences of iron overload on the liver include fibrosis and cirrhosis that can be exacerbated by the presence of underlying liver disease *(260*, *321)*. The incidence of HCC in hereditary hemochromatosis (HH) is increased over 100× relative to a comparative control population *(260*, *331)*. Outcomes in heterozygotes for HFE seem similar to wildtype, except for those 1–2% individuals who are compound heterozygotes with C263Y/H63D *(332*, *333)*. The odds ratio of HCC in HFE C282Y carriers or homozygotes is 3.5, while it is 7 in those with cirrhosis indicating that HFE is a risk factor for HCC *(333)*. The HCC population is enriched for C282Y carriers than is found in the general population indicating a possible risk factor for its development and progression *(332–334)*. The increased risk from HFE alleles is found in alcoholic cirrhosis and some cases of HCV viral hepatitis, but not HBV viral hepatitis patients *(322*, *332*, *334)*. Animal models of liver disease in combination with iron overload also demonstrate an increase in disease progression. For example, transgenic mice overexpressing the HCV polyprotein fed a diet enriched in iron develop microvesicular steatosis indicative of mitochondrial damage and impaired energy use with fatty acid retention and earlier onset of HCC than their

littermates similar to those humans that develop fatty liver with HCV infection *(335)*. A wide range of hepatic tumor phenotypes is observed in HFE *(336)*. Interestingly, a high incidence of p53 mutations has been observed in one series of HCC from HFE patients *(337)*. Importantly, epigenetic defects are observed in liver tissue from 75% of the HFE patients examined prior to the onset of cirrhosis with hypermethylation and hence gene expression decreases *(338)*.

Wilson's disease or inherited copper overload disease can result in cirrhosis, hepatitis, and HCC. Wilson's disease is found in 1:30,000 with a carrier rate of 1:250 *(339)*. Cerruloplasmin is decreased in the serum of Wilson's disease patients. This autosomal recessive disorder is due to a mutation in the P-type ATPase responsible for biliary copper excretion (ATP7B) located in the trans-Golgi network *(340)*. The most prevalent mutation, H1069Q, is observed in 30% of Wilson's patients of European decent. Other mutations of the ATP7B gene exist and can also result in Wilson's disease *(339)*. In addition, modifier genes that impact the severity of the disease also exist. Copper is normally ingested and absorbed through the GI tract and excreted through the bile. Copper is transported in the serum bound to histidine. Copper binds to glutathione or metallothionein, and cerruloplasmin. It is excreted into the bile in part through a secretory pathway involving ATP7B. The Long Evans Cinnamon rat is susceptible to non-viral hepatitis with subsequent formation of liver neoplasms, the male is more susceptible to the development of liver tumors *(341*, *342)*. The LEC rat is a model of Wilson's disease that develops a non-viral hepatitis due to copper overload. These rats also have disturbances in iron metabolism. Those animals that survive the hepatitis will develop HCC. The toxic milk mouse has a mutation in M1356V and G712D, has defects in copper transport *(343)*, and a knock-out mouse (ATP7B) has also been generated *(344)*. If intracellular copper accumulates beyond the ability of the hepatocyte to buffer it, then hepatic damage will ensue with copper release into the circulation and its accumulation in other tissues.

5.7.2. Alpha-1 AntiTrypsin

Alpha-1 antitrypsin (AAT) is a prevalent protease inhibitor (Pi) found in the plasma *(345)*. The most prevalent mutation is a Glu342Lys caused by a G to A transition called the Z mutation *(346*, *347)*. Adult males that are homozygous for the Z mutation (PiZZ) have an increased risk of cirrhosis and HCC *(346–348)*. Alpha-1 antitrypsin results in an increased risk of HCC in the absence of cirrhosis in homozygotes *(348)*. Carriers (PiZ) are also believed to be at an increased risk for HCC *(349)* especially in combination with other risk factors *(350*, *351)*. While the mechanism of α1AT alleles on disease etiology is unclear, the altered protein structure may

induce the unfolded protein response. Alternatively, this acute phase serum protein, which acts as an inhibitor of elastase and is synthesized by the liver and macrophage is retained in the liver resulting in a plasma insufficiency. Retention in the liver and consequent polymerization can result in cirrhosis and to HCC *(346*, *347)*.

5.7.3. Hereditary Tyrosinemia

Tyrosinemia is an autosomal recessive disorder that can lead to HCC. This inborn error of metabolism results *(352)* from inactivation of fumarylacetoacetate hydrolase (FAH) resulting in the buildup of its substrate fumarylacetoacetate (FAA) and malylacetoacetate (MAA). As a consequence, these individuals excrete high levels of succinylacetone into the urine *(352)*. MAA and more specifically FAA have multiple effects on liver cells including apoptosis, ER stress response, redox balance including GSH depletion, and cell cycle arrest. Since the last step in the catabolism of tyrosine is blocked, tyrosine is elevated in the serum. These patients have a rapid conversion from micro to macronodular cirrhosis and later conversion to dysplasia and HCC. Without pharmacological (nitisinone) treatment or now surgical intervention, the prognosis was poor with acute liver failure predominant, followed by HCC *(353*, *354)*. A mouse model has been developed in which FAH is knocked out *(355)*. This mutant recapitulates the pathogenesis of human hereditary tyrosinemia type 1 and can be protected by nitisinone *(356)*. Intervention with nitisinone does not reverse gene expression changes associated with tyrosinemia *(357)*. Thus, pharmacological treatment can delay, but may not prevent HCC development. Genetic manipulation reversal of double mutant FAH mice formed through ENU mutagenesis do not develop preneoplastic lesions or HCC, suggesting that the lack of complete reversal of the phenotype by pharmacological intervention is due to incomplete blockage of the formation of toxic intermediates *(358)*.

5.7.4. Citrullinemia

The inborn errors of disease associated with the urea cycle *(359*, *360)*; namely, mutation of arginosuccinate results in acute liver toxicity *(361)*. Citrullinemia type I is an autosomal recessive disorder that is caused by a deficiency in the rate-limiting enzyme in the urea cycle, argininosuccinate synthetase (ASS1). In severe cases, a hyperammonia can occur that is fatal neonatally. An argininosuccinic aciduria with an increase in citrulline and ammonia in the serum is observed. Since citrulline is essential in nitrogen homeostasis, disruption of ammonia removal results in toxicity to the liver. There is a broad mutational pattern and each genotype has different phenotypes *(361)*. A knock-out mouse has been generated that has high citrulline blood levels and a severe hyperammonemic phenotype *(362*, *363)*. The aspartate–glutamate carrier (AGC), SLC25A13, gene

mutations result in citrin deficiency *(364)* and may develop hepatic steatosis and steatohepatitis *(365)*. These type 2 citrullinemia patients have an increased level of pancreas-derived trypsin inhibitor and are associated with pancreatitis *(364)*. A decrease in this mitochondrial ACG, citrin, results in hepatic apoptosis through a caspase pathway in which the bax to bcl2 ratio is inverted *(366)*. A knock-out model has been described, but does not recapitulate all of the pathologies associated with adult-onset type 2 citrullinemia *(367)*. The citrin/mitochondrial glycerol-3-phosphate dehydrogenase double knock-out mutant is a better model for type 2 citrullinemia *(368)*. Urea cycle disruption and perturbations of nitrogen removal can have adverse effects on the liver as exemplified by citrullinemia.

5.8. Summary

Chemicals from a variety of chemical classes can initiate, promote, and lead to the development or progression of HCC. The effects of chemical agents occur on the backgrounds of a variety of genetic alterations and diseases. Animal models have proven invaluable in the assessment of the early pathogenesis of primary liver cancer by chemicals. The late stage neoplasms analyzed from the human demonstrate that multiple etiologies, molecular pathways, and genetic changes accompany neoplastic development in the liver. Combinations of genetic factors, environmental exposures, and background liver disease will be modeled in increasing complex ways in the future to better recapitulate the role of chemicals in HCC development and progression. Systems biology tools as applied to the pathogenesis of HCC will be informative about the pathways that chemicals dysregulate in different genetic and disease backgrounds to lead to HCC development and progression.

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4 Molecular Mechanisms of Hepatocellular Carcinoma: Insights to Therapy

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CONTENTS

ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most frequently occurring human malignancies in the world and is associated with a high mortality rate. As such, understanding the molecular underpinnings of this cancer in order to identify novel diagnostic markers, therapeutic targets, and prognostic indicators that aid in patient care is a major goal for clinicians and researchers alike. Progress has been made on this front over the past several years resulting in the development of drugs that specifically target processes believed to propagate HCC cell transformation, growth, and metastasis such as cell surface receptor–ligand interaction and signal transduction, cell cycle and apoptosis progression, extracellular matrix remodeling, vasculogenesis, motility, histone modification, and others. Many of these agents

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have been assessed in pre-clinical animal models and are now being evaluated in human clinical trials in the United States and elsewhere. This chapter will discuss targeted therapies for HCC under study in humans as well as the pathways they intercept.

Key Words: Hepatocellular carcinoma; gene expression; clinical trials; targeted therapies; molecular mechanisms

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is an aggressive malignant tumor of the liver that accounts for about 80% of primary hepatic cancers in adults *(1)*. HCC is now the fifth most common type of malignancy and the third leading cause of cancer mortality worldwide *(2)*. The underlying determinants of HCC are diverse and include a variety of viral, toxic, and metabolic insults, most of which result in cirrhosis *(3)*. Populations from certain geographic regions such as Asia and Africa suffer disproportionately from HCC reflecting a high incidence of hepatitis B virus (HBV) infection and aflatoxin exposure *(3)*. However, the number of HCC diagnoses has been rising in low-incidence areas such as the United States, Western Europe, and Japan, likely due to an increase in hepatitis C virus (HCV) infection in these populations *(3)*. Over 20,000 new diagnoses of liver cancer in Americans were expected to be rendered in 2008 *(4)*. Without appropriate screening, HCC comes to clinical presentation late in its course when surgical intervention is no longer an option; at any stage, this tumor is notoriously resistant to standard systemic chemotherapy as a result of innate tumor resistance as well as underlying liver disease making it a difficult malignancy to manage clinically *(5)*. Due to HCC's aggressive behavior, insidious presentation, resistance to therapy, and general prevalence, a concerted global effort has been put forth over the past two decades to dissect the molecular mechanisms of HCC in order to reveal clues to diagnosis, therapy, and prognosis. Because of these Herculean efforts, novel diagnostic markers, therapeutic targets, and prognostic indicators for HCC have been discovered, and although the clinical utility of many of these newly identified molecular hallmarks must still be rigorously assessed, it has become evident that some discoveries may well constitute 'medical breakthroughs.'

Most major risk factors for HCC (such as HBV or HCV infection, aflatoxin exposure, alcohol abuse or metabolic derangements like hereditary hemochromatosis) *(6)* cause sustained hepatocyte damage by one mechanism or another and incite repair. However, the healing process in the liver may be incomplete rendering hepatocytes vulnerable to additional assault.

Cycles of hepatocyte death and replication promote fibrous deposition, cirrhosis, hepatic insufficiency, and outgrowth of pre-neoplastic and frankly malignant clonal cell populations *(1)*. DNA damage that accumulates in cell clones may result from replication errors inflicted by aberrant cell cycle transit, direct mutagenesis, oxidative stress triggered by inflammation, or a combination of these mechanisms *(7)*. Because HCC risk factors are so varied, each is capable of eliciting unique pro-tumorigenic alterations substantiating the notion that, despite falling under the same histologic classification, HCCs are, in fact, quite heterogeneous *(8)*. To this end, molecular 'signatures' reflecting the inciting cause or recurrence pattern have been identified *(9)*. However, it is also obvious that some molecular mechanisms are activated in the majority of liver tumors, regardless of the underlying risk factor. It is these candidates, in particular, that make attractive therapeutic targets.

It has been proposed that transformation of a normal hepatocyte into one with malignant potential requires at least five or six individual genetic insults *(10)*. Numerous studies have been carried out comparing normal, cirrhotic, dysplastic, malignant, and metastatic liver tissues *(11,12)* in attempts to categorize the genetic mutations associated with each step leading toward malignancy. Due to HCC's inherent heterogeneity, however, this has been a difficult task. Depending on the type of molecular tool or test employed (e.g., classic cytogenetics *(13)*, CGH *(14)*, SNP *(15)*, expression *(12)*, or microRNA arrays *(16)*, or proteomic approaches *(17)*) and the type of tissue tested (i.e., normal vs. tumor, dysplastic vs. malignant, solitary vs. multifocal tumors, invasive vs. non-invasive tumors, HBV+ vs. HCV+ tumors, or mouse vs. human tumors), HCCs can be subclassified into a multitude of different categories. However, one intriguing HCC subclassification which has been further substantiated in human and rodent HCC *(18–20)* separates tumors into two groups: those with mutant p53 and genomic instability and those with beta-catenin aberrancy and cancer gene hypermethylation.

p53 is a multifaceted transcription factor that is crucial to inducing cell cycle arrest and eliciting apoptosis *(21)*. Dysregulation of p53 in HCC occurs through a combination of loss of heterozygosity (LOH) observed in over half of HCCs (57%–8/14) *(22)* and mutation detected in about 28% of HCC cases worldwide *(23)*. In addition, upregulation of cellular and viral factors that bind and sequester p53, such as mdm-2 *(24,25)* and hepatitis B virus X protein (Hbx) *(26,27)*, is seen in HCCs. Another key role of p53 is to maintain DNA integrity *(21)* which is commonly lost in human HCC. Genomic instability in HCC is characterized by non-random DNA losses on chromosomes 1p, 4q, 6q, 8p, 10q, 13q, 16p, 16q, and 17p and gains of genomic material on chromosomes 1q, 5p, 5q, 6p, 7q, 8q, 17q, and 20q *(28)*. Specific chromosomal losses and gains correlate with the underlying risk factor and tumor differentiation *(11)*.

Beta-catenin is a multifunctional protein, the ultimate purpose of which is to control gene transcription. It acts as a conduit linking signaling at the plasma membrane where beta-catenin normally resides with the nucleus where it transactivates a repertoire of gene targets, many of which are proto-oncogenes including c-myc and cyclin D1. Under normal conditions, soluble extracellular signals such as Wnt ligands, extracellular matrix interactions, Met transmembrane receptor, and other determinants control beta-catenin activation and localization *(29)*. However, in HCC, several molecular mechanisms leading to abnormal beta-catenin activation, such as beta-catenin gene mutation seen on average in about 22% of human HCCs *(23)*, downregulation of E-cadherin *(30,31)*, or PIN1 overexpression *(32)* bypass normal control steps thus leading to excessive gene expression driven by beta-catenin.

Aberrant DNA methylation is an epigenetic event usually described in the context of neoplasia: HCC is no exception. In hepatic and other tumors, DNA methylation alterations are characterized by a state of global demethylation and focal de novo hypermethylation of CpG islands in specific gene promoters. These changes can result in stimulation of proto-oncogenes and silencing of tumor-suppressor genes *(33)*. Interestingly, it has been postulated that DNA hypermethylation alterations in the liver may well reflect normal physiologic responses to aging and to inflammation. As compared to normal liver from younger patients, aged livers, livers with active hepatitis, and HCC tissues showed stepwise increases in DNA hypermethylation of a set of epigenetic markers *(34)*. Taken together, these findings regarding beta-catenin activation and global hypermethylation in HCC suggest that inhibition of key tumor-suppressor genes (via hypermethylation) in combination with mutation of oncogenes (like beta-catenin) can incite HCC development in the absence of large-scale genomic alterations.

In addition to p53 and beta-catenin, a host of factors have been linked to the malignant transformation, growth, or invasion of liver cancer. They fall into several categories such as cell surface receptors and their ligands, intracellular effector molecules, cell cycle and apoptosis regulators, extracellular matrix remodeling agents, vasculogenic factors, motility inducers, histone modifiers, and telomerases. As a wonderful testament to humankind's ingenuity and the power of scientific research, several targeted therapies have been designed to modulate the activity of some of these pathways. Many have since been assessed in pre-clinical models and are now being evaluated in human clinical trials across the world (Table 1). The remainder of this chapter will focus on those signaling pathways with agents showing therapeutic potential in HCC and engendering clinical enthusiasm.

Table 1
Select Targeted Therapies for HCC Currently Under Evaluation in Clinical Trials (www.clinicaltrials.gov)*

Pathway	*Target*	*Agent*	*Class*
Growth factors/receptor tyrosine kinases	EGFR/ErbB2	*Gefitinib*	Kinase inhibitor
		Erlotinib	Kinase inhibitor
		Lapatinib	Kinase inhibitor
		Cetuximab	Anti-EGFR mAb
		Trastuzumab	Anti-ErbB2 mAb
	VEGF/VEGFR-1/-2/-3	*Cediranib*	Kinase inhibitor
		Sunitinib	Kinase inhibitor
		Brivanib	Kinase inhibitor
		Vandetanib	Kinase inhibitor
		Pazopanib	Kinase inhibitor
		ABT-869	Kinase inhibitor
		IMC-1211B	Anti-VEGFR-2 mAb
		Bevacizumab	Anti-VEGF mAb
	IGF1R	*IMC-A12*	Anti-IGF1R mAb
Intracellular signal transducers	Ras	*Lonafarnib*	Farnesyltransferase inhibitor
	Raf/Mek/Erk/MAPK	*Sorafenib*	Kinase inhibitor
		AZD6244	Kinase inhibitor
	mTOR	*Sirolimus*	Binds FKBP-12; inhibits mTORC1
		Everolimus	Binds FKBP-12; inhibits mTORC1
	Abl/Src	*Dasatinib*	Kinase inhibitor
Transcription factors	RAR-alpha	*TAC-101*	Inhibitor
Cell cycle modulators	p53	*Ad5CMV-p53*	Gene therapy
	CDK	*Flavopiridol*	Inhibitor
Pro-survival molecules	Survivin	*LY2181308*	Anti-sense oligomer

(*Continued*)

Table 1
(Continued)

Pathway	*Target*	*Agent*	*Class*
DNA modification enzymes	HDAC	*Belinostat*	Inhibitor
Inflammation modulators	Cox-2	*Celecoxib*	Inhibitor
Proteasome	26S Proteasome	*Bortezomib*	Inhibitor

Abbreviations used: CDK—cyclin-dependent kinase; EGFR—epidermal growth factor receptor; HDAC—histone deacetylase; IGF1R—insulin-like growth factor1 receptor; mAb—monoclonal antibody; VEGF(R)—vascular endothelial growth factor (receptor). *—at the time of writing.

2. RECEPTOR TYROSINE KINASES AND THEIR LIGANDS

In order to adapt to changes in the surrounding environment and sense the needs of the host organism, cells must be able to receive and act upon signals from the extracellular milieu. Such communication is facilitated by a variety of mechanisms; one of these is through receptor tyrosine kinases (RTKs) anchored in the plasma membrane. Through their extracellular domains, RTKs bind protein ligands with high specificity and affinity and, following engagement, emit potent intracellular cues that regulate cell division, motility, survival, and a number of crucial cellular activities. Because of their capacity to control cell growth, RTK signaling is tightly governed and short lived. Steps to ensure that RTK signal emission is of proper intensity and duration include limiting RTK–ligand interaction, promoting RTK internalization and degradation as well as activating phosphatases and other measures *(35)*.

A specific set of RTKs have garnered attention in the study of liver cancer. Some are activated by well-established hepatic mitogens. These RTKs and their ligands include the epidermal growth factor receptor (EGFR) and its family members which bind EGF, transforming growth factor alpha (TGF-alpha) and other EGF-related ligands, and Met, the RTK for hepatocyte growth factor (HGF). These particular RTKs and their ligands are often overexpressed in HCC and are thought to help drive malignant hepatocyte replication, invasion, and motility. Other RTKs are involved in tumor neovascularization such as the vascular endothelial growth factor receptors (VEGFRs). HCCs are highly vascular tumors and secrete factors like VEGF to promote vessel ingrowth in order to establish and maintain an oxygen-rich blood supply.

Because RTKs and their relatives, the intracellular tyrosine kinases (such as src, abl, JAK, and others), are such powerful transducers of malignant transformation, growth, and invasion, they were among the earliest candidates to be explored for their therapeutic targeting potential. To that end, imatinib, an inhibitor with specificity for the bcr-abl oncogene product resulting from a t9;22 chromosomal translocation observed in human chronic myelogenous leukemia, was one of the first rationally designed targeted small molecule therapeutics approved by the US Food and Drug Administration (FDA) to treat cancer *(36)*. Since then, numerous inhibitors with specificity for other tyrosine kinases have been developed, and their efficacy in pre-clinical models and clinical trials for various types of cancer including HCC (Table 1) is under investigation.

2.1. Epidermal Growth Factor Receptor Family and Ligands

The EGFR family of receptors contains four members: EGFR (ErbB1 or Her1), ErbB2 (Her2 or neu), ErbB3 (Her3), and ErbB4 (Her4). Although ErbB2 is an orphan receptor with no known ligand, activation of its tyrosine kinase domain is facilitated by heterodimerization with and transphosphorylation by other EGFR family members *(37)*. Two of the four EGFR family members that bear relevance to HCC are EGFR and ErbB2, the most well characterized in hepatocyte biology and HCC being EGFR itself and its associated ligands, EGF and TGF-alpha. Ligand-activated EGFR promotes hepatoctye motility *(38)* and morphogenesis *(39)* and contributes to liver regeneration *(40)*.

With regard to liver cancer, TGF-alpha mRNA *(41)* and protein *(42–44)* are overexpressed in human HCCs, particularly in HBV+ cases, as compared to adjacent liver tissue. In addition, transgenic mice overexpressing TGF-alpha in the liver develop HCC after a year *(45,46)*. On the other hand, results of studies examining EGFR expression in liver cancer are conflicting with some showing increased EGFR expression in HCCs *(47,48)* while others not *(49,50)*. Perhaps a more relevant observation is that enhanced tyrosine phosphorylation of EGFR at residue Y845 was noted in 72% (13/18) of HCC tissues using Western blot *(51)*. However, two studies did not detect EGFR mutation in human HCC samples *(52,53)*.

Data supporting a role for ErbB2 in human HCC are limited. Ito et al. *(48)* demonstrated that 21% of HCCs expressed ErbB2, while others did not observe ErbB2 expression in liver cancers *(54)*. Mutation of the kinase domain in the ErbB2 gene (*her2/neu*) occurs in some solid tumors such as non-small cell lung carcinoma (NSCL) *(55)*. An analysis of human HCCs for ErbB2 mutation did not reveal the presence of those gene variants previously described by others in NSCL cancer but did identify a novel amino

acid change (H878Y) the authors propose could alter ErbB2 activity in 11% (2/18) of HCCs tested *(52)*.

Several targeted inhibitors of EGFR and ErbB2 are currently under study in clinical trials for HCC (Table 1). Results of Phase II clinical trials of erlotinib, an orally active inhibitor of EGFR, and cetuximab, an anti-EGFR monoclonal antibody administered intervenously, in patients with advanced liver cancer have been published. In the first of two reports of erlotinib efficacy, about a third (32%) of patients showed no disease progression at 6 months with erlotinib therapy while 9% of patients demonstrated a partial radiologic response. However, over a quarter (26%) of patients in the study required erlotinib dose reductions due to skin toxicity and diarrhea *(56)*. Patients in the second erlotinib study did not show evidence of radiologic response to the treatment but over 40% demonstrated progression-free survival at 16 weeks of therapy *(8)*. Phase II trials with cetuximab were less promising revealing that the median progression-free survival for patients on treatment was 1.4 months despite the drug being well tolerated *(57)*.

2.2. Vascular Endothelial Growth Factor Receptor Family and Ligands

Solid tumors require new blood vessel formation or neovascularization in order to enlarge *(58)*; this is clearly the case with HCC *(59)*. The portal circulation serves as the blood supply for early HCCs; however, as tumors expand, their oxygen demands increase. As a consequence, the oxygen-enriched hepatic arterial supply is tapped to feed the tumor *(59)*. The vascular endothelial growth factors consisting of six members (VEGF-A through -E and placenta growth factor [PLGF]) *(59,60)* and their receptors are essential to this process. Three tyrosine kinase cell surface receptors exist for VEGF including VEGFR-1 (flt-1), VEGFR-2 (KDR or flk-1), and VEGFR-3 (flt-4). The activities of VEGF-A, VEGF-B, and PLGF appear to be mediated primarily through VEGFR-1, while VEGF-A and -E utilize VEGFR-2, and VEGF-C and -D bind VEGFR-3 *(61)*.

VEGF expression is upregulated in most cases of human HCC *(62–65)*. Some studies indicate, however, that VEGF protein levels are elevated to a greater extent in non-tumorous adjacent cirrhotic tissue than HCCs *(66,67)*. Expression of both VEGFR-1 and -2 mRNA has been detected in human liver tumors; however, one study showed that, of the two, VEGFR-1 mRNA levels were greater in tumor tissues *(62)*, whereas another determined that VEGFR-2 transcripts were more abundant in HCCs *(68)*. Liu et al. *(69)* determined that human HCC cell lines express both VEGFR-1 and -2 by flow cytometric analysis and Western blot and that cell proliferation was augmented by addition of VEGF to the cultures. These findings point to the

possibility that, in addition to a paracrine effect of VEGF on endothelia to promote neovascularization in HCC, a VEGF/VEGFR autocrine circuit may also exist to stimulate growth of liver tumor cells.

A multitude of agents targeting the VEGF/VEGFR axis are available and in clinical trials for HCC. They include tyrosine kinase inhibitors, an anti-VEGF monoclonal antibody (bevacizumab), and an anti-VEGFR-2 antibody (IMC-1211B, see Table 1). Recently, a Phase II clinical trial assessing the efficacy of bevacizumab in combination with GEMOX (gemcitabine–oxaliplatin) in patients with advanced HCC was completed and results released *(70)*. In this study, CT perfusion scan was used to monitor tumor blood flow, blood volume, permeability surface area, and mean transit time as a means of tracking tumor vascularity pre- and post-treatment *(70)*; the degree of tumor contrast enhancement which can be assessed by CT has been shown to correlate with tumor neovascularization in HCC *(71)*. Bevacizumab therapy was significantly associated with longer mean transit time indicating increased tumor capillary leakiness. In addition, the results showed that the percent change in mean transit time following bevacizumab treatment correlated with patient outcome. Median progression-free survival was 5.3 months in this study *(70)*.

2.3. Insulin-Like Growth Factor Receptor-I and Ligands

The insulin-like growth factors-I and -II (IGF-I and -II) stimulate hepatocyte replication *(72)* and appear to be involved in human liver tumorigenesis. IGFs can engage three types of receptors: the insulin receptor (IR), IGF1R, and IGF2R/mannose-6-phosphate receptor. The first two are RTKs; the latter is not. Of the three, only IGF1R binds IGFs with high affinity and thus likely propagates most IGF-induced signaling *(73)*. The majority of evidence implicates IGF-II over IGF-I in human HCC. Several studies have shown that the human IGF-II gene is genomically imprinted in normal adult tissues *(74)* except in the liver: normal hepatic tissue expresses IGF-II from both of its alleles *(75)*. However, in HCC, biallelic IGF-II expression ceases *(76,77)*, and usage of a fetal-type IGF-II promoter recommences *(78,79)*. This is accompanied by increased IGF-II protein and mRNA expression in human HCCs *(78,79)*. To this end, a Phase II clinical trial to determine the efficacy of the anti-IGF1R monoclonal antibody known as IMC-A12 in those with advanced HCC recently began recruiting patients *(80)*.

3. INTRACELLULAR SIGNAL TRANSDUCERS

Numerous and diverse intracellular signaling molecules serve to receive and amplify cues emitted from cell surface receptors. They deliver them to intended recipients such as the mitochondria, the nucleus and other key

organelles, cellular structures, and proteins. In some cases, the signal 'hand off' between intracellular molecules occurs in a relatively orderly and predictable fashion—from one pathway member to the next, and so on; however, as more insight into these cascades is obtained, it is becoming clear that branch points, nodes, and various deviations along the signaling chain occur and complicate our understanding. The messages these cellular liaisons transport are certainly consequential to the well-being of the host; thus their pathways are highly regulated at multiple levels, in order to maintain normal cell function *(81)*. Because of their crucial role in governance of cell signal transduction, several of these signaling proteins and their respective pathways are mutational targets in cancer.

Some of the better known intracellular signaling molecules targeted in human liver cancer include beta-catenin (as described) and c-myc *(82,83)*. The ras GTPase, while historically a proven player in rodent hepatocarcinogenesis *(84)*, is now gaining significance as a mediator in human HCC as well. Additional factors recently linked to HCC are PI3K pathway constituents (p110alpha and PTEN) and members of the rho GTPase cascade. Pharmacologic inhibitors of several intracellular signaling pathways are now being tested in human HCC patients (Table 1). The following section will focus on a subset of the pathways with targeted therapies under clinical evaluation for liver cancer.

3.1. The Small GTPase Superfamily

Ras and rho are members of the small GTPase superfamily. Their localization to the inner plasma membrane is facilitated by farnesyl and palmitoyl lipid moieties attached to their protein backbone. Ras and rho are active when bound to GTP and, in this state, recruit signal transducers (such as raf in the case of ras). To become inactive, these small GTP_{ases} hydrolyze GTP to GDP. Several adaptor and regulatory proteins such as guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) positively and negatively regulate ras and rho. Some of these adapters, such as Grb2 and Sos (a rasGEF), link stimulated RTKs to the small GTPases leading to their activation thus initiating signaling cascades which influence cell proliferation, cytoskeletal rearrangement, and expression of genes such as cyclin D1, $p21^{WAF1/CIP1}$, and $p27^{KIP}$*(85)*.

A large body of evidence demonstrates that ras is involved in normal hepatocyte replication in culture *(86)* and in vivo *(87,88)*. The ras family consists of three major isoforms: H-, K-, and N-ras. Mutation of these isoforms has been detected in several types of cancer *(89)*. In the case of human liver cancer, about 33% (6/18) of vinyl chloride-associated HCCs

were found to harbor K-ras mutations. Incidentally, such mutations were also detected in surrounding non-tumorous liver tissue in two of the six cases *(90)*. Other than in the instance of vinyl chloride-induced liver tumorigenesis, K- or H-ras mutations have rarely been detected in human HCCs *(91–94)*. Despite a lack of data implicating ras gene mutation as a common cause of human HCC, the ras signaling cascade may be upregulated in this tumor through other mechanisms. One such mechanism may be due to suppressed expression of a ras effector molecule and suspected tumor suppressor known as ras association domain family 1A (RASSF1A). The gene promoter region of RASSF1A is hypermethylated in 93% of human HCCs (14/15). Aberrant methylation of RASSF1A was also seen in human livers with fibrosis (2/2) and cirrhosis (3/4), but not in normal liver (0/2) *(95)*, suggesting that ras pathway signaling provides a permissive environment promoting hepatocyte replication and accumulation of additional genetic alterations. Similar RASSFIA methylation differences were observed by others *(96,97)*.

The expression and activity of several factors involved in the rho cascade are also deranged in human HCC. These include rhoA, rhoC, and deleted in liver cancer-1 and -2 (DLC-1 and -2). The rho subfamily of GTPases can be subdivided into six smaller groups based on structural similarity: rhoA and rhoC, along with rhoB, comprise one of these six groups *(98)*. Recently, a study demonstrated that rhoA mRNA and protein levels were 2.0- and 2.7-fold higher, respectively, in tumor tissue than adjacent liver. These observations correlated with tumor invasion and poor histologic differentiation. The authors concluded that overexpression of rhoA is associated with a poor prognosis *(99)*, a finding supported by another group *(100)*. Wang et al. *(101)* examined human HCCs for gene mutation and mRNA expression of rhoC. They found no mutations in rhoC in any samples, but they did observe that intrahepatic and invasive/metastatic HCCs expressed 1.8- and 3.3-fold more rhoC mRNA, respectively, than adjacent liver tissues leading them to postulate that rhoC may be involved in liver tumor cell invasion and metastasis, an idea backed by others *(102)*.

Human chromosome 8p, in particular 8p21.3-22 *(103)*, is a deletion hotspot in HCC, and its loss is associated with metastasis *(104)*. The DLC-1 gene has been cloned from this region and encodes a novel rhoGAP *(105)*. About half of HCCs show LOH in the DLC-1 gene *(106)*. Others demonstrated loss of DLC-1 gene expression in about 20–67% of human liver tumors *(106,107)*. Decreased DLC-1 expression may be due to DLC-1 gene hypermethylation which has been observed in 24% (6/25) of HCCs as compared to adjacent liver *(106)*. The DLC-2 gene, encoding a rhoGAP related to DLC-1, has been cloned from chromosome 13q12.3 *(108)*. This region is also commonly deleted in HCC *(109)*. DLC-2 mRNA levels were reduced in 18% (8/45) of liver tumors compared to adjacent liver. Functional studies

demonstrated that DLC-2 preferentially regulates rhoA and another small GTPase cdc42 *(108)*. A Phase II clinical trial of lonafarnib, an orally available farnesyltransferase inhibitor that inhibits farnesylation of ras and rho, is underway for patients with primary liver cancer *(80)*.

3.2. Raf-Mek-Erk/MAPK Pathway

The raf, mek, and erk/MAPK serine/threonine kinases make up the core transducers in the mitogen-activated protein kinase (MAPK) signaling cascade, a network which controls cell proliferation, motility and survival *(110)*. Research has revealed that the once simple 'linear pipeline' concept of MAPK signal transduction is no longer valid. The observations that a variety of raf, mek, and erk/MAPK isoforms and regulatory molecules (such as Sprouty proteins) exist and that compartmentalization of various pathway constituents and effectors occurs complicate the scheme *(110)*.

The raf-mek-erk/MAPK pathway is activated in cultured hepatocytes after growth factor stimulation *(38)* and during liver regeneration *(111)*. Upregulation of the pathway is also seen in human liver tumors. Schmidt et al. observed that mek and erk/MAPK isoforms were significantly overexpressed in human HCC tissues as compared to adjacent liver tissue by Western blot; in addition, they determined that erk/MAPK protein levels correlated with increased erk/MAPK kinase activity in the tumor samples *(112)*. Elevated erk/MAPK expression *(113)*, phospho-erk/MAPK levels *(114)*, and erk/MAPK activity *(115,116)* in HCCs were also noted by others.

Sprouty (Spry) proteins and SPREDs (Sprouty-related proteins with an Ena/vasodilator-stimulated phosphoprotein homology-1 domain) are newly discovered negative regulators of the raf-mek-erk/MAPK pathway. Sproutys reside in the cytosol until they are recruited to the inner plasma membrane following RTK activation. There, they partner a variety of scaffolding proteins and signal transduction molecules, including raf itself, to control signal propagation of the raf-mek-erk/MAPK cascade *(117)*. SPREDs appear to function in a similar manner *(118)*. Recently, HCCs were examined for Sprouty-2 (Spry2) expression: 73% of tumors (8/11) expressed significantly less Spry2 mRNA than non-tumor liver tissue. However, neither LOH at the Spry2 locus nor hypermethylation of the Spry2 gene promoter was detected to account for the dampened expression *(119)*. Yoshida and coworkers *(116)* observed that mRNA expression of either SPRED-1 or -2 was downregulated in 84% (27/32) of HCCs as compared to adjacent liver. In over two-thirds of those cases (68%, 22/27), repression of both SPRED-1 and -2 mRNA levels was noted *(116)*.

Two small molecule inhibitors that target the raf-mek-erk/MAPK cascade are presently under clinical investigation for human HCC. The first, and least characterized, is AZD6244, an orally available drug that targets mek. Recruitment for a pair of Phase II clinical studies examining AZD6244 in advanced HCC is proceeding *(80)*. The second inhibitor of the raf-mek-erk/MAPK pathway to be studied in humans with HCC is sorafenib, a multikinase inhibitor with activity directed against raf and certain cell surface RTKs. Promising preliminary results of a randomized, double-blind, multicenter Phase III clinical trial examining the efficacy of sorafenib vs. placebo in patients with advanced HCC (the SHARP study) have been released *(120,121)*. These data prompted the US FDA to approve sorafenib use for HCC in late 2007 and to recommend it as a first-line therapy in patients with advanced, unresectable HCC with mild to moderate liver impairment (Child-Pugh class A or B) *(122)*, thus making sorafenib the first targeted therapeutic for HCC to obtain FDA approval.

Findings from the SHARP study revealed that treatment with sorafenib was associated with an increased median time to progression from 2.8 months with placebo to 5.5 month with therapy. Over 60% of patients on sorafenib demonstrated progression-free survival at 4 months compared to only 42% in those receiving placebo. However, no complete responses were noted in the treatment group, and only 2.3% of those treated with sorafenib showed a partial response as compared to 0.7% of patients receiving placebo *(120,121)*, suggesting that sorafenib stabilizes, rather than cures, advanced HCC *(120)*. Several additional clinical trials of sorafenib in HCC are underway *(80)*.

4. OTHER THERAPEUTIC TARGETS: PRESENT AND FUTURE

Most targeted therapeutics under evaluation in human HCC have been developed against RTKs and their immediate downstream signal effectors; however, treatments directed toward other molecular targets are also being tested. Some of the more noteworthy include those which inhibit proteasomal degradation (bortezomib) and histone deacetylation (belinostat).

The proteasome comprises a large multi-subunit drum-shaped enzymatic complex that degrades damaged or excessively abundant proteins. Protein substrates destined for proteasomal degradation are tagged with ubiquitin, a small protein marker of about 8 kDa in size, by one of several ubiquitin E3 ligases. The relative abundance of a variety of proteins is managed by the proteasomal pathway. Evidence suggests that proteasomal blockade in cancer cells, including HCC, increases their susceptibility to undergo apoptosis *(123)*. One mechanism sensitizing HCC cells to apoptosis may be due

to upregulation of receptors for the death ligand, Trail, and to increased DISC formation *(124)*. The clinical efficacy of proteasomal inhibition with bortezomib was confirmed in the treatment of multiple myeloma *(125)*. Currently, Phase II trials of bortezomib in patients with advanced HCC are underway *(80)*.

Acetylation or deacetylation at specific terminal lysine residues in histones impacts chromatin structure, gene promoter access, and transcriptional regulation by promoting chromatin accessibility or condensation, respectively. Histone deacetylases (HDACs) are responsible for removing acetyl groups from terminal lysine residues in histones, thus allowing DNA to compact into heterochromatin repressing gene transcription. HDACs are increasingly recognized as important contributors to tumorigenesis; as such, HDAC inhibitors have been developed which, among other activities, lead to reactivation of pro-apoptotic gene expression and suppressed cancer cell growth in culture *(126)*. For example, in human HCC cell lines, exposure to the HDAC inhibitor trichostatin-A resulted in cell cycle arrest, apoptosis, and hallmarks of hepatocyte differentiation *(127)*. Upregulated HDAC expression has been observed in human liver tumors and correlated with a higher incidence of portal vein invasion and poor histologic differentiation *(128)*. One HDAC inhibitor belinostat completed a Phase I clinical trial in patients with advanced solid tumors and showed a favorable toxicity profile, a dose-dependent effect on HDAC activity and disease stabilization in over a third (39%) of patients *(129)*. Recruitment for a Phase II clinical trial of belinostat in patients with advanced HCC is ongoing *(80)*.

One molecular target in HCC primed for clinical assessment is the receptor tyrosine kinase Met, the ligand of which is HGF. Clinical trials with three different Met inhibitors are progressing, mostly for solid tumors including pancreatic and gastric carcinoma, but as of now, no trials specifically geared toward liver cancer have been initiated *(80)*. As mentioned earlier, the HGF-Met axis is a highly relevant hepatic signaling system. Its function is paramount to hepatic development *(130–132)*, hepatocyte replication, motility *(38)* and survival *(133,134)*, and to liver regeneration *(40)*. In addition, Met dysregulation is seen in most human HCCs *(135–137)*, and its overexpression is associated with the presence of intrahepatic metastases and poor patient outcome *(136)*. Met dysfunction in human HCC can also occur through activating mutations in the Met gene *(138)*. Overexpression of HGF in human liver tumors has not been a consistent finding *(137,139)*, but enforced overexpression of HGF in hepatocytes is oncogenic in a mouse model *(140)*.

The next decade should give the oncology community the necessary time to determine whether targeted small molecule therapeutics work well to stabilize or cure HCC. More likely than not, combination therapies, either as cocktails of molecularly targeted treatments or as mixtures of conventional

cytotoxic agents and targeted drugs, will yield the greatest clinical benefit for liver cancer patients with unresectable disease. The outcomes of these studies are eagerly awaited.

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5 Genomic Profiling of Human Hepatocellular Carcinoma

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CONTENTS

ABSTRACT

Numerous studies of human gene function have been launched since the sequencing of the human genome. Global molecular profiling studies of hepatocellular carcinoma (HCC) are providing a comprehensive view of the expression changes that occur during the carcinogenic process and are uncovering promising biomarkers with clinical potential. In this chapter, an overview of recent gene expression profiling of human HCC is provided along with a summation of the mechanistic, diagnostic, and prognostic significance of these findings. Emerging concepts associated with these studies are also addressed and biomarkers present in serum are highlighted. Current

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profiling studies, conducted on multiple array platforms, are powerful tools which have provided useful clues to begin to unravel the mechanisms of HCC biology and improve clinical outcome.

Key Words: Hepatocellular carcinoma; molecular marker; gene expression profiling; microarray; liver disease

1. HEPATOCELLULAR CARCINOMA: CLINICAL CONCERNS

The wide heterogeneity of HCC and the complexity of its diagnostic and prognostic assessment (dependent on tumor grade/residual liver function) have interfered with clinical recommendations and progress. Despite many studies of HCC, the specific changes associated with its development remain ill defined and there is no clear consensus on which of the many different staging systems introduced around the world is best *(1–4)*. Although individuals at high risk for HCC development are routinely screened by ultrasonography and serum α-fetoprotein (AFP), most patients are diagnosed at advanced disease stages. AFP evaluation, however, can be non-specific, varies significantly between ethnic groups, and is only observed in a HCC subgroup with small tumors *(5)*. Although several additional serum proteins have been suggested to improve HCC diagnosis, they lack sensitivity and specificity and await confirmatory studies or development of quantitative methods to evaluate their utility *(6, 7)*. It is possible that a single marker may not be sufficient to diagnose HCC and as such, it may be important to test combinations of markers to improve diagnostic performance. HCC diagnosis with the AFP marker, therefore, remains the gold standard and improvement of the current screening system is an imperative goal. Liver function impairment and the expression of multidrug resistance genes render HCC treatment especially difficult *(8)*. Since most HCC patients are diagnosed at an advanced stage, they are often excluded from potentially curative therapies such as resection and liver transplantation. Eligibility for resection (relatively good liver function and small tumors) or transplantation (Milan criteria/limited donor livers/long waiting list) is also quite slim and post-surgical survival is complicated by a predominant occurrence of tumor recurrence/metastasis *(9–15)*. Methods to improve survival include percutaneous ethanol injection, radiofrequency ablation, and transarterial chemoembolization (TACE) *(16)*.

The current status of HCC emphasizes the importance of understanding the underlying biology of this disease and the development of new screening and treatment stratification programs to refine diagnosis and improve patient outcome. Relevant biomarkers to assist HCC diagnosis and prognosis are particularly essential at early HCC stages and can be used as

novel therapeutic agents. The identification of such biomarkers in a high-throughput fashion is now possible through the advent of global molecular profiling.

2. GENE EXPRESSION PROFILING: CURRENT TECHNOLOGIES

The gene expression profile of a particular cell type or tissue has been analyzed by using multiple technologies including differential screening of cDNA libraries, subtractive cDNA hybridization, differential display of RNA, and serial analysis of gene expression (SAGE). More recently, global expression profiling studies have been conducted using platforms consisting of genes (cDNA/OLIGO microarrays), noncoding RNA, proteins (proteome arrays), tissues (tissue microarray), and genetic aberrations (arrayCGH/methylation) *(17)*. Although previous methodologies to study HCC have advanced the field, gene expression profiling of clinical samples from HCC patients and HCC-related cell lines has enriched the breadth of HCC knowledge and has allowed researchers to begin to tackle some of the key disease-related concepts that still remain.

2.1. Microarray Platforms

Microarrays provide genomic information and insight into biological processes on a genome-wide scale. Their miniaturized ordered arrangement of targets (nucleic acids/proteins/tissues) located at defined positions on a solid support (platform) enables high-throughput parallel analysis of many targets by specific hybridization. The composition of an array platform can be global (an entire genome on a slide) or specific (pathways, cell/tissue type) and allows for the characterization of a transcriptome/proteome/genome. A brief overview of widely used array platforms is provided below.

2.1.1. Expression Arrays (cDNA/OLIGO/Noncoding RNA)

The cDNA microarray reports differences in gene expression levels between samples and functions on the basis of specific and high-affinity molecular recognition between complementary cDNA strands (PCR-derived cDNA or 20–60mer OLIGO fragments) representing exonic regions of the genome *(18)*. The regulation of mRNAs can be analyzed using microRNA (miRNA) arrays, which globally interrogate the expression of small endogenous (21–35 nt) noncoding RNAs. Platforms that detect mature and precursor forms of >500 miRNAs are now commercially available *(19–21)*.

2.1.2. Protein Arrays (Proteome/Tissue)

Although mRNAs are transcribed, they may not be translated and thus mRNA copy number may not reflect the number of functional protein molecules in a cell. Thus, proteome arrays may provide a better view to understand gene function. Protein function or protein detecting arrays involve immobilization of antibody probes to detect antigens in a sample, or vice versa. These arrays can be used to quantify proteins, determine post-translational modifications, and correlate proteins with disease advancement or with certain treatments/environments *(22)*. Tissue microarrays (TMA) allow tissue-based profiling using small cylinders of formalin-fixed tissues arrayed in a single paraffin block *(23)*. Protein arrays are limited by the protein concentration range required for direct detection within a given sample and current instrumentation allows for only a fraction of the proteome to be examined. The measurement of low-abundance targets also remains a challenge, but high-affinity probes, such as SELEX (systematic evolution of ligands by exponential enrichment) aptamers, can help to resolve this problem *(24, 25)*.

2.1.3. Genomic Arrays (CGH/Methylation)

Array comparative genomic hybridization (aCGH) using the BAC-based (bacterial artificial chromosome) and the more recent oligonucleotide-based CGH enables high-resolution multi-loci mapping of small genomic regions with copy number changes, such as amplification or deletion *(26, 27)*. BAC aCGH is limited by costly, time-consuming, low-yield clone production and noisy data due to non-specific hybridization of repetitive sequences. Oligonucleotide aCGH allows for flexibility in probe design, greater genomic coverage, and higher resolution (~50 kB). New tiling BAC arrays, however (where each BAC overlaps with its contiguous BAC), can increase resolution, signal intensity, and more accurately define the boundaries of genomic aberrations, but require a high concentration of high-quality BAC DNA for good array performance *(28, 29)*. Recently, a few CGH array studies have been followed by bisulfate DNA sequencing or methylation-specific PCR to identify HCC-related epigenetic changes.

2.2. Microarray Analysis

Methodologies for microarray analysis can be either unsupervised or supervised *(30–32)*. Unsupervised methods attempt to characterize the components of a data set without a priori input or knowledge of a training set. Internal structures or relationships in data sets are found by feature determination which groups genes with interesting properties (principal component analysis), cluster determination which groups genes or samples with similar patterns of gene expression (nearest-neighbor clustering,

self-organizing maps, k-means clustering, and one- and two-dimensional hierarchical clustering), and network determination which graphs gene–gene or gene–phenotype interactions (Boolean networks, Bayesian networks, and relevance networks). On the other hand, supervised methods are used to determine genes that fit a predetermined pattern. This technique finds genes with expression levels that are significantly different between groups of samples (e.g., cancer classification) and can be used to find genes that accurately predict a characteristic of that sample (e.g., survival or metastasis). The significance found by supervised methods has been evaluated using parametric, non-parametric, and analysis of variance procedures which involve permutations, random partitioning of the studied data set, and false discovery limits. These methods are employed to assess the validity of signatures associated with a tested feature and to rule out the identification of a signature by random chance. Several criteria exist for determining differential expression, including absolute or ratio of expression levels across samples and subtractive degree of change between groups. These methods include the nearest-neighbor approach, decision trees, neural networks, and support vector machines. A gold standard has been proposed for analysis of array studies which involves the use of a training data set to initially identify a signature, a test data set to assess its predictive/classification capacity, and an independent set for validation studies.

3. HCC MICROARRAY STUDIES: EMERGING CONCEPTS

Microarray studies have provided vast amounts of information concerning the genes, proteins, and genomic changes that occur in HCC-related disease. These investigations have revealed changes that occur across a spectrum of cirrhosis, HCC tumors, HCC subtypes, epigenetic alterations, and progressive phenotypes (metastasis/recurrence). A summary of these signatures, affected pathways, and diagnostic/prognostic markers is provided in Table 1. An overview of these studies along with a synopsis of emerging perspectives gleaned from these analyses is provided in this section.

3.1. Diagnostic HCC Signatures

3.1.1. Chronic Liver Disease Signatures

HCC develops largely in a previously diseased liver, contributed by chronic liver disease (CLD). CLD has been attributed to hepatitis viral attack, genetic/metabolic disorders, alcohol abuse, and/or environmental influences *(13, 33)*. The HCC population is, therefore, quite heterogeneous, since the tumor and CLD can be at different evolutionary stages at diagnosis, each with different therapeutic perspectives and survival probabilities.

Table 1
HCC Microarray Signatures[a]

			Study design					
Platform	*Prediction signatures*	*Sample size*	*Training set*	*Testing set*	*Independent set*	*Validation method*	*Affected pathways*	*Ref*
Genome	6 genes	20 cases	Yes	No	No	No	No	*(78)*
	13 regions	104 HCCs; 76 non-HCCs	Yes	No	No	No	No	*(230)*
	4 gain regions; 7 lost regions	19 HCV–HCCs	Yes	No	No	No	No	*(77)*
	673 clones	44 cases; 5 HCC cell lines	Yes	No	No	No	No	*(76)*
	2 prognostic regions	87 cases	Yes	No	No	FISH	No	*(81)*
	8 gain regions; 9 LOH regions	36 cases	Yes	No	No	No	No	*(231)*
	7 genes	88 cases	Yes	No	No	qRT-PCR; ISH	No	*(109)*
	3 regions	63 HCCs; 4 HCC cell lines	Yes	No	No	qRT-PCR; WB; IH	Etiology specific	*(96)*

Table 1
(Continued)

			Study design					
Platform	*Prediction signatures*	*Sample size*	*Training set*	*Testing set*	*Independent set*	*Validation method*	*Affected pathways*	*Ref*
cDNA	HCC patho/ virological genes	20 cases	Yes	No	No	No	Wide range	*(68)*
	2253 genes	4 cases	Yes	No	No	RT-PCR	Cell cycle; Wnt signaling	*(40)*
	9 HCC genes; 22 differentiation genes	10 cases	Yes	No	No	No	No	*(63)*
	HCC-specific genes	102 HCCs; 74 non-HCCs; 7 BLTs; 10 MCs; 10 HCC cell lines	Yes	No	No	No	P53; Vascular invasion	*(113)*
	HCV-cirrhosis genes	6 HCV–HCCs; 4 AIHs; 8 NLs	Yes	No	No	RT-PCR	Wide range	*(232)*

(*Continued*)

Table 1
(Continued)

Platform	Prediction signatures	Sample size	Study design				Affected pathways	Ref
			Training set	Testing set	Independent set	Validation method		
	HCC subgroup genes	18 HCCs and 1 HB cell line	Yes	No	No	No	AFP	*(101)*
	HCC patho/ virological genes	15 cases	Yes	No	No	RT-PCR	Wide range	*(67)*
	90 clones	22 HCC foci	Yes	No	No	No	No	*(114)*
	83 genes	14 HBV–HCCs; 31 HCV–HCCs	Yes	No	No	No	No	*(45)*
	HCC-specific genes	8 cases	Yes	No	No	RT-PCR	No	*(57)*
	50 genes	20 HCV–HCC cases	Yes	Yes	Yes	RT-PCR; NB	No	*(58)*
	30 genes	30 cases	Yes	Yes	Yes	RT-PCR; IH; WB	No	*(122)*
	83 genes	17 WT-p53 cases; 5 MT-p53 cases	Yes	No	No	RT-PCR	P53	*(66)*

Table 1
(Continued)

Platform	Prediction signatures	Sample size	Study design				Affected pathways	Ref
			Training set	Testing set	Independent set	Validation method		
	3 genes	33 cases	Yes	Yes	Yes	No	Wide range	*(135, 136)*
	68 genes	37 cases	Yes	Yes	Yes	RT-PCR	Wide range	*(61)*
	59 genes	10 cases	Yes	Yes	No	No	No	*(62)*
	220 genes	20 HCCs; 17 non-HCCs; 31 NLs	Yes	Yes	Yes	No	No	*(55)*
	89 HBV–HCC /9 HCV–HCC genes	14 HBV–HCCs; 31 HCV–HCCs	Yes	No	No	RT-PCR	No	*(93)*
	30 genes	14 HCCs; 7 HBVs; 11 HCVs; 3 HHCs; 5 WDs; 16 PBCs; 10 ALDs; 7 AIHs	Yes	Yes	Yes	No	No	*(90)*
	20 genes	100 cases	Yes	Yes	No	No	No	*(129)*

(*Continued*)

Table 1
(Continued)

Platform	Prediction signatures	Sample size	Study design				Affected pathways	Ref
			Training set	Testing set	Independent set	Validation method		
	406 genes	91 HCCs; 60 non-HCCs; 18 NLs	Yes	Yes	No	No	Cell cycle; Apoptosis	*(119)*
	668 genes	7 Solitary large HCCs; 15 Nodular HCCs	Yes	No	No	RT-PCR; WB; IH	No	*(233, 234)*
	129 genes	12 LC nodules; 5 HCCs	Yes	Yes	No	No	No	*(91)*
	HCC subtype genes	43 HCCs; 3 HCC cell lines	Yes	No	No	RT-PCR; NB	Apoptosis; Immune response	*(100)*
	44 genes	33 HCCs; 23 non-HCCs	Yes	Yes	Yes	RT-PCR	No	*(59)*
	4 genes	33 cases	Yes	Yes	Yes	RT-PCR; IF	Immune response	*(235)*
	240 genes	50 hepatocellular nodules	Yes	Yes	No	No	No	*(105)*
	Chromosome-specific HCC genes	31 HCCs; 19 non-HCCs	Yes	No	No	qRT-PCR	No	*(236)*

Table 1
(Continued)

			Study design					
Platform	*Prediction signatures*	*Sample size*	*Training set*	*Testing set*	*Independent set*	*Validation method*	*Affected pathways*	*Ref*
	38 genes	20 cases	Yes	No	No	No	No	*(51)*
	Liver-fibrosis genes	3 non-HCV cases; 19 HCV cases	Yes	No	No	RT-PCR	No	*(92)*
	25 genes	13 LCs; 23 non-tumor LCs near HCC; 19 HCCs	Yes	No	Yes	qRT-PCR	No	*(44)*
	63 genes	6 HCAs; 8 well-differentiated HCCs	Yes	Yes	Yes	qRT-PCR; IH	No	*(102)*
	HBV–HCC genes	15 HCCs; 5 non-HCCs	Yes	Yes	No	qRT-PCR	c-myc	*(237)*
	HCC subgroup genes	61 human HCCs; 39 mouse HCCs; Rat fetal hepatoblasts; Rat adult hepatocytes	Yes	Yes	No	No	AP-1 activation	*(142)*

(*Continued*)

Table 1
(Continued)

Platform	Prediction signatures	Sample size	Study design				Affected pathways	Ref
			Training set	Testing set	Independent set	Validation method		
	36 genes	40 cases (28 SNs, 12 MNs)	Yes	Yes	No	No	No	*(60)*
	31 genes	24 cases	Yes	No	No	RT-PCR	Cell cycle; Immune response	*(103)*
	14 genes	18 cases	Yes	Yes	Yes	qRT-PCR	No	*(130)*
	35 genes	35 cases	Yes	No	No	qRT-PCR; WB	No	*(238)*
	46 genes	35 cases	Yes	No	No	RT-PCR	Cell adhesion	*(134)*
	123 genes	2 $HBV^{-}HCV-$ HCCs; 2 HBV−HCCs; 2 HCV–HCCs; 6 non-HCCs	Yes	No	No	No	Cell cycle; Cell adhesion	*(118)*
	40 genes	1 NIN HCC; 3 dysplastic nodules; 3 HCCs	Yes	No	No	RT-PCR	No	*(104)*

Table 1
(Continued)

Platform	Prediction signatures	Sample size	Study design				Affected pathways	Ref
			Training set	Testing set	Independent set	Validation method		
	17 genes	115 cases	Yes	Yes	Yes	qRT-PCR; IH	Immune response	*(138)*
	217 genes	40 cases	Yes	No	No	NB; IH	No	*(115)*
	Angiogenesis soluble factors	38 HCV–HCCs; 52 HCV–LCs; 6 NLs	Yes	No	No	qRT-PCR	Angiogenesis	*(50)*
	5 genes	218 cases	Yes	Yes	Yes	qRT-PCR; ELISA	No	*(54)*
	57 genes	48 cases	Yes	Yes	Yes	qRT-PCR	Wide range	*(137)*
	248 genes	40 cases	Yes	No	No	RT-PCR; NB; IH	No	*(239)*
	2 genes	40 cases	Yes	Yes	Yes	qRT-PCR	Wnt	*(144)*
microRNA	8 miRNAs	24 HCCs; 22 non-HCCs	Yes	No	No	NB	No	*(89)*
	35 miRNAs	17 HCCs; 21 LCs	Yes	No	No	qRT-PCR; NB	No	*(95)*
	15 miRNAs	3 cases	Yes	No	No	qRT-PCR; NB	No	*(240)*
	35 miRNAs	17 HCCs; 21 LCs	Yes	No	Yes	qRT-PCR; NB	No	*(95)*

(*Continued*)

Table 1
(Continued)

			Study design					
Platform	*Prediction signatures*	*Sample size*	*Training set*	*Testing set*	*Independent set*	*Validation method*	*Affected pathways*	*Ref*
	40 miRNAs	10 cases (without virus)	Yes	No	No	NB	No	*(241)*
	20 miRNAs	131 cases	Yes	Yes	Yes	qRT-PCR	No	*(123)*
	22 miRNAs	19 cases	Yes	No	No	qRT-PCR	No	*(86)*
Proteome	32 proteins	30 HCCs; 15 NLs	Yes	No	Yes	WB	No	*(73)*
	250 features	20 CLDs; 38 HCCs	Yes	Yes	No	No	No	*(97)*
	90 proteins	67 cases; 12 NLs	Yes	No	Yes	WB; IH	Cell growth; angio-genesis	*(71)*
	4 peptides	34 HCC serums; 39 F1/F2 fibrosis serums; 44 F4 fibrosis serums	Yes	No	No	No	No	*(99)*
	11 spots	20 cases	Yes	No	No	WB	No	*(69)*
	15 signals	25 HCCs; 23 HCC margins; 28 NLs;	Yes	No	No	IH	No	*(72)*

Table 1
(Continued)

Platform	Prediction signatures	Sample size	Study design				Affected pathways	Ref
			Training set	*Testing set*	*Independent set*	*Validation method*		
	12 peaks	26 SNs; 45 MNs; 6 HCCs	Yes	No	No	No	No	*(41)*
	6 proteins	28 HCC serum; 18 normal serum	Yes	No	Yes	WB	No	*(242)*
	11 peaks	41 HCC serums; 51 HCV–LC serums	Yes	No	No	No	No	*(98)*
Others								
Genome/ regional expression bias	2 regions	39 HCCs	Yes	No	No	No	No	*(82)*

(*Continued*)

Table 1
(Continued)

Platform	*Prediction signatures*	*Sample size*	*Study design*				*Affected pathways*	*Ref*
			Training set	*Testing set*	*Independent set*	*Validation method*		
Genome/ cDNA array	Correlation between genomic copy and cDNA level	41 HCCs; 12 HCC cell lines	Yes	No	No	PCR	No	*(79)*
Genome/ cDNA array	31 positive-correlated genes	20 cell lines	Yes	No	No	qRT-PCR	No	*(83)*
Proteome/ cDNA array	93/125 correlated proteins	14 cases	Yes	No	No	No	No	*(243)*

Note

[a] The papers cited in this table are microarray data performed on clinical HCC samples.

AIH—Autoimmune hepatitis; ALD—alcoholic liver disease; BLT—benign liver tumor; CLD—chronic liver disease; HB—hepatoblastoma; HCA—hepatocellular adenocarcinoma; HCC—hepatocellular carcinoma; HHC—hemochromatosis; LC—liver cirrhosis; LOH—loss of heterozygosity; MC—metastatic cancer; MN—multinodular; MT—mutant-type; NIN—nodular in nodular; NL—non-disease liver; PBC—primary biliary cirrhosis; SN—single nodular; WD—Wilson's disease; WT—wild type.

ELISA—Enzyme-linked immunosorbent assay; IH—immunohistochemistry; ISH—in situ hybridization; NB—northern blot; RT-PCR—reverse transcription polymerase chain reaction; qRT-PCR—quantitative RT-PCR; WB—: Western blot.

Several gene expression profiling studies have focused on CLD etiologies (mainly of hepatitis B and/or C viral infection) in order to identify diagnostic markers, particularly for early detection. cDNA arrays have shown that genes associated with the TH1 immune response (including lymphocyte/monocyte activation), fibrosis, extracellular matrix remodeling, cell–cell interactions, proliferation, cell growth regulation, and apoptosis are upregulated in HCV–CLD *(34–36)*. Candidate genes ($n = 260$) involved in signal transduction pathways, cell cycle control, metastasis, transcriptional regulation, immune response, and metabolism were aberrantly expressed under HBx induction by cDNA array *(37)*. In our laboratory, we have shown that primary hepatocytes expressing HBx have altered expression of several cellular oncogenes and tumor-suppressor genes *(38)*. Oncogenes, cell cycle regulators, intracellular transducers, stress response genes, apoptosis-related genes, and transcription factors were also shown to be upregulated in response to HBV infection, while growth factors were downregulated *(39)*. Several of these HBV-altered genes were correlated to regions with amplification (1q, 8q, 13q) or loss of heterozygosity (4q, 8p, 16q, 17p) *(40)*. In addition, global proteomic profiling has shown that cirrhotic nodules in a HBV background contain signatures associated with clonal expansion *(41)*.

The differentially expressed genes altered by HBV and HCV infection have also been analyzed using microarrays. Differential gene expression was shown by cDNA array between chronic HBV and HCV hepatic lesions, with HBV-affecting genes related to inflammation while HCV-affected genes related to the anti-inflammatory process *(42)*. However, only a slight difference between HBV and HCV host cell infection was found in another cDNA array, but the authors noted that the differentially expressed genes were clearly regulated in a reciprocal manner *(43)*. Other cDNA studies have shown that lectin and cytochrome p450 can distinguish viral cirrhosis subtypes *(44)*. In an OLIGO-based study, 83 genes were found to differ between HBV and HCV–HCC, including those related to signal transduction, metastasis, and immune response *(45)*. Another OLIGO array study revealed 176 genes that were altered upon HBV or HCV viral infection, including the interferon-inducible gene IFI27 *(46)*. IFI27 was also shown to be highly upregulated in HCV–HCC in an OLIGO array-based study in our laboratory in which human hepatocytes were infected with HBV- or HCV-related genes *(47)*. OLIGO arrays have also shown that an HCV-specific gene (NS5A) can modify pathways associated with cell motility and adhesion, lipid transport and metabolism, calcium homeostasis and regulate the immune response through NF-kB signaling *(48, 49)*. The strongest effects were a downregulation of an adenylate synthetase (OAS-69) and an upregulation of IL8 which both affect IFN anti-viral activity. In a proteomic array study, angiogenic factors, including VEGF, were upregulated in HCV–HCC tissues *(50)*.

Taken together, these observations suggest that a high degree of changes take place in CLD tissues. The identification of these premalignant changes may be useful to classify patients with CLD groups or those patients at risk for developing HCC. In addition, these notable changes involved in CLD may be useful for early detection and thus provide a window of opportunity to intervene with an effective therapy. These studies have also demonstrated that some genes are consistently altered in preneoplastic conditions and HCC, highlighting early changes that may also play a role in disease progression. Many of these studies, however, involve relatively small cohorts, identify relatively large signatures/classifiers, do not provide sufficient follow-up data to confirm patient outcome, or are not validated in independent cohorts. Therefore, large prospective studies and/or meta-analysis of existing data sets will be needed to validate the potential clinical use of these CLD-related markers as diagnostic tools.

3.1.2. Tumor Biomarkers (Tumor vs Non-tumor)

Microarray studies have also enhanced our understanding of how the HCC process alters the regulatory network of genes and proteins in a way that differs from the respective normal tissue or disease-free samples. For example, cDNA analysis of HCC vs normal samples has found 38 differentially expressed genes while HBV-related cell lines revealed signatures (356 genes) composed of upregulated ribosomal-related genes *(51, 52)*. TIPUH1, a regulator of transcription and RNA processing of growth control genes, has also been shown to be upregulated in HCC by cDNA array *(53)*. In our laboratory, we have shown that five genes (GPC-3, PEG10, MDK, SERPINI1, and QP-C) are elevated in HCC samples, even in those with low AFP status compared to normal tissue *(54)*. A cDNA array of non-HBV/HCV-infected HCC vs normal tissues revealed 61 differentially expressed genes *(55)*. A number of studies have also found alterations in genes involved in protein synthesis, growth factors, oncogenesis, stress, inflammation, cell proliferation, transcription, protein degradation, p53, Wnt/β-catenin, metabolism, and tumorigenesis pathways in HCC *(40, 56–62)*. Similar studies have shown that activators of neutrophils, antiapoptotic genes, interferon response genes, and proteins related to cell differentiation or development are differentially expressed in HCV–HCC *(63)*. Integrin and Akt/NF-kB signaling were also upregulated in HCC along with a serum biomarker (CSTB) using cDNA arrays *(64, 65)*. OLIGO arrays have shown that p53-related genes ($n = 83$) are affected by HCV infection and alter immune response, transcription, transport, signal transduction, and metabolism in tumors *(66)*. Several of these pathways, along with growth factor alterations, were found in cDNA arrays comparing HBV- or HCV-positive tumor with non-tumor

tissue *(67)*. A clear distinction was found between HBV and HCV samples, where HBV-affected genes were involved in apoptosis, p53, and the G1/S transition while HCV-affected genes were more heterogeneous. In a separate cDNA array study, upregulation of mitosis-promoting genes was observed in the majority of HBV or HCV tumors vs non-tumors while differentially expressed genes between HBV and HCV tumors encoded enzymes that metabolize carcinogens and/or anticancer agents associated with malignant/invasive phenotype, apoptosis, or immune regulation *(68)*.

Proteomic and TMA arrays have also been used to address the differences that occur following tumor formation. A proteomic analysis of human HCV-related HCC found alterations in glycolysis enzymes, mitochondrial β-oxidation pathways, and cytoskeletal proteins when compared to non-tumor tissue *(69)*. Other HCC-related protein classifiers include proteins involved in heat shock response, glycolysis, fatty acid transport and trafficking, amino acid metabolism, cell cycle regulation and cell stress, and metabolism-related enzymes *(70–72)*. Other upregulated genes in HCC include insulin growth factor-II, metalloproteases, signal transducers and activators of transcription (STAT), suppressors of cytokine signaling, and cyclin D1 while collagens and SMAD pathways were downregulated *(73)*. A TMA study of HCC/non-tumor comparisons found HCC-specific expression of the transcription repressor zinc fingers and homeoboxes 2 (ZHX2) protein expression which correlated with differentiation stage *(74)*.

Multiple studies have aimed to determine HCC-related regions of genetic gain or loss. Most studies have found similar regions of gain (1p, 4q, 8p, 13q, 16q, and 17p) and loss (1q, 6p, 8q) in HCC *(75–77)*. In addition, a study of 120 HCC samples found LOH at 6q and 9p in small, well-differentiated tumors *(75)*. A comparison of tumor vs non-tumor HCC samples using BAC aCGH included frequent DNA copy number gains of 20q and found that high Jab1 levels correlated with chromosome 8q gain in HCC *(76)*. A study of HCV-associated HCC revealed that increases of DNA copy number were frequent at 10p while decreases were frequent at 10q *(77)*. These authors found increases in copy numbers of the LAMC2, TGFB2, and AKT3 genes (located on 1q) and decreases in copy numbers of FGR/SRC2 and CYLD (located on 1p and 16q, respectively) in tumors. In a study of 20 HCC cases, oncogenes were amplified in 1q, 8p, and 11q regions while loss occurred at 13q and 4q *(78)*. In a study of HBV-infected HCC, gains on 1q, 6p, 8q, 9p were observed while losses in 1p, 16q, and 19p occurred in most patients *(79)*. Midorikawa et al. showed a frequent gain of 1q, 8q, 12q, 17q, and 20q as well as a loss of 4q, 8p, 13q, and 17p in HCC *(80)*. Gains in regions encoding MET, c-myc, and FGF4 were also found in a CGH study of HCC while a separate study identified narrow regions of frequent amplification on chromosome 1p, frequent deletion on 17q, and alterations in 7q21 encoding paternally expressed 10 (PEG10) *(81–83)*.

miRNAs have recently been utilized as potential HCC diagnostic markers. Expression profiling studies have defined the liver-specific miR-122 to be highly downregulated in HCC tumors and cell lines *(84)*. miRNA array studies have also demonstrated that miR-21 can contribute to HCC growth and spread by modulating PTEN *(85)*. In other miRNA-based studies, mir-224, a 16-miRNA set, and a novel mRNA-like noncoding RNA named highly upregulated in liver cancer (HULC) were found to be significantly upregulated in HCC *(86–88)*. In another study comparing HCC samples and adjacent non-tumor, eight miRNAs were shown to be significantly altered, five of which were downregulated in HCC and could predict HCC with 97% accuracy *(89)*.

3.1.3. Tumor Biomarkers (Tumor vs Cirrhosis)

Array-based comparisons have also been made between early neoplastic stages (fibrosis/cirrhosis) and HCC. A study of 59 preneoplastic CLDs (hepatitis, autoimmune hepatitis, primary biliary cirrhosis, etc.) conducted in our laboratory found genes associated with high or low risk of HCC development *(90)*. This 273-gene signature was validated in three independent cohorts and included 12 secretory genes in the top gene set. In separate cDNA array-based studies, 25 cirrhosis-specific genes were identified that were related to inflammatory status of adjacent HCC tissue and 129 genes were altered in HCC compared to liver cirrhosis samples *(44, 91)*. In an OLIGO array-based study of fibrosis, carbohydrate metabolism genes were elevated in HCC patients when compared to cases with F3-4 fibrosis *(92)*. In a comparison of HCC with CLD (either HBV or HCV positive) or HCC without CLD in an OLIGO array, genes involved in transcription, metabolism, and cell growth were differentially expressed *(93)*. An RT-based study of cirrhosis vs HCV–HCC showed that twelve genes were significantly altered (including GPC3, TERT, survivin, XLKD1, and CDH1) *(94)*. MiRNA platforms have also demonstrated that 35 miRNAs including let7 and miR-181 family members differ between HCC and cirrhosis *(95)*. aCGH of 63 HCCs found etiology-dependent copy number gains, including 8q24 and MYC overexpression in viral and alcohol-related HCCs *(96)*. The use of comprehensive proteomic profiling of sera to differentiate HCC from CLD found 250 significantly different proteins, while an 11-peak SELDI profile or 4-peptide panel could distinguish HCC from HCV-related cirrhosis and was an independent predictor of HCC *(97–99)*.

3.1.4. Tumor Biomarkers (Tumor Subtype Signatures)

Several HCC array studies have also compared HCC tumors to identify subtypes or to compare various tumor stages or nodular status to understand the changes that occur between early and late tumorigenesis. In a cDNA study of HCC and HCC cell lines, two subgroups of HCC were

identified that were related to either IFN-associated inflammation or apoptosis, while another cDNA study composed of 19 HCC cell lines found 2 subtypes that were correlated with AFP expression *(100, 101)*. In a comparison of multinodular and solitary HCC, cDNA arrays revealed 230 genes that were specific to multinodular recurrence, while only 36 were commonly expressed *(60)*. A separate cDNA study of HCCs from 10 patients found several genes related to histological subtype *(62)*. In an OLIGO study of well-differentiated HCC vs hepatocellular ademonas, 63 genes were found to be differentially expressed, demonstrating molecular differences despite similarities in morphology *(102)*. Another OLIGO study identified 31 genes that differed between early and advanced HCV–HCCs *(103)*. In other OLIGO-based studies analyzing nodule-in-nodule HCC, dysplastic nodules, and HCCs, the authors found 40 genes involved in the transition from dysplasia to early-stage tumors and 240 genes that could accurately classify tumors according to histological grade *(104, 105)*.

3.1.5. Tumor Biomarkers (Epigenetic Signatures)

HCC development is thought to be a multistep process involving not only accumulation of genetic changes but also epigenetic changes, such as methylation, which can reversibly alter regulatory genes. A few studies have begun to address the epigenetic changes that occur in HCC. In a cDNA/bisulfite PCR study, the demethylating agent 5-Aza-dC was used to identify hepatocyte growth factor (HAI-2/PB) as a frequent hypermethylated gene in HCC *(106)*. In another cDNA array and bisulfite PCR study, insulin-like growth factor-binding protein was found to be hypermethylated and down-regulated in HCC *(107)*. An OLIGO-based analysis of human HCC cell lines showed that treatment with 5-Aza-dC resulted in a decrease of the tissue factor pathway inhibitor TFPI-2 *(108)*. In addition, Pang et al. found a loss of an unmethylated 6q allele in HCC encoding a putative tumor-suppressor gene *(109)*. However, in a study of 60 primary HCCs using aCGH and methylation-specific PCR, a causal relationship was not observed between the methylation status of nine CpG islands, including p16, COX2, and APC, and patient outcome *(110)*.

Thus, numerous array studies have shown that multiple tumor-specific alterations occur during hepatocarcinogenesis. A detailed exploration of these changes may offer new insight regarding HCC biology and provide avenues for diagnostic advances. Within platform types, however, marker sets are quite different from one another, despite a similarity in comparison groups which could be due to platform makeup, sample heterogeneity, differences in etiology or ethnicity among samples. In addition, many of these studies lack validation and are only drawn from a rather small data set, and therefore further studies will be needed to determine whether the identified changes can be widely useful for diagnostic or HCC classification purposes.

In sum, these studies clearly demonstrate that measurable changes occur during HCC development that may be useful for early detection.

3.2. Prognostic HCC Signatures

3.2.1. Metastasis/Survival/Recurrence Signatures in HCC Tumor or Non-tumor Tissues

Metastasis and recurrence are major factors affecting the outcome of patients with HCC. Understanding the mechanisms involved in the process of tumor invasion and metastasis is a major challenge. Biomarkers related to these processes may have clinical prognostic utility. Important questions related to metastasis involve initiation, the relationship between primary and metastatic tumors, and whether these metastatic changes are inherent to the cell or are acquired through time and/or environmental status. The current metastasis model suggests a multistage carcinogenic process initiated by rare genetic alterations in a single cell, followed by clonal selection and population expansion *(111)*. In HCC, however, such stepwise and specific progression-related genetic changes have not been illustrated *(3)*.

The transcriptome, proteome, and genome of metastatic HCC cells have been studied using array technology. Comprehensive cDNA analysis of HCV-related HCCs has identified 35 genes involved in portal vein invasion (PVI) including the inhibitor of DNA binding 2 (ID2), encoding a liver-rich dominant-negative helix–loop–helix protein which was validated by qRT-PCR, Western blot analyses, and in an independent set *(112)*. A 91-gene vascular invasion signature was also found in a separate cDNA study and 90 clones were correlated with intrahepatic metastasis in a study of 22 HCC foci *(113, 114)*. Another cDNA study of HCC found 217 genes associated with differentiation status and metastasis, including ANXA2 *(115)*. A cDNA array was also employed to profile gene expression patterns in two subtypes of HCC, solitary large HCC (SLHCC) and nodular HCC (NHCC), which differ significantly in metastatic incidence *(116)*. A significant decrease in RhoC expression in SLHCC compared to NHCC was strongly correlated with HCC metastasis, implicating RhoC as a potential prognosis marker and therapeutic target for HCC *(117)*. Another cDNA-based study found that HCC with high expression of ubiquitin-conjugating enzyme, Ube2c, displayed PVI and poor disease-free survival rates while 906 genes were found to differ between HCC and surrounding tissue, generating clusters (A and B) that were associated with patient survival *(118, 119)*. OLIGO array studies have also shown that MAPK pathway and angiogenesis factors such as VEGF and HGF are associated with HCV–HCC while 39 genes were significantly correlated with metastasis, including cortactin, a cortical actin-associated protein substrate of Src *(50, 120, 121)*. In our laboratory,

we have applied cDNA arrays to show that intrahepatic metastatic lesions are indistinguishable from their primary HCC, while primary metastasis-free HCC was distinct from primary HCC with metastasis *(122)*. These data indicate that primary HCC with metastatic potential is an inherent quality of the primary tumor rather than a capability acquired over time through mutation. The 153-HCC metastasis gene signature, whose lead gene was osteopontin (OPN), could accurately classify metastatic HCC. In our laboratory, we have also investigated whether certain miRNAs are associated with HCC metastasis *(123)*. We identified a unique 20-miRNA metastasis signature that could significantly predict ($p < 0.001$) primary HCC tissues with venous metastases from metastasis-free solitary tumors. A survival risk prediction analysis revealed that a majority of the metastasis-related miRNAs were associated with survival. Furthermore, the 20-miRNA tumor signature was validated in 110 additional cases as a significant independent predictor of survival ($p = 0.009$) and was significantly associated with survival and early-stage HCC. These 20 miRNAs may provide a simple profiling method to assist in identifying HCC patients who are likely to develop metastases/recurrence.

TMAs and aCGH have also been used to study HCC metastasis. The clinical significance of FGF3 overexpression was studied by TMA in 60 pairs of primary/metastatic HCCs and showed that overexpression of FGF3 was significantly associated with HCC metastasis and recurrence ($p < 0.01$) *(124)*. ZHX2, described earlier as a possible HCC diagnostic marker, was also found by TMA to be expressed significantly higher in primary lesions with metastasis than in those without this phenotype *(74)*. A significant overexpression of clusterin (CLU) was found in metastatic HCC in a paired tissue study ($n = 104$), and Id-1 (inhibitor of differentiation/DNA synthesis) and also Rac and VEGF, key angiogenic factors in cancer progression, were correlated with HCC metastasis by TMAs *(125, 126)*. Meanwhile, aCGH array analysis of early and advanced components of nodule-in-nodule HCC found that genetic inactivation of the APC gene played a significant role in the progression of sporadic HCC, possibly through activation of the Wnt/β-catenin pathway *(127)*. Another study revealed that loss of 17p13.3 and 8q11 was an independent prognostic indicator of poor HCC patient survival *(81)*. LOH has also been observed at 16q and 17q in HCC and occurred more frequently in metastatic lesions *(128)*. aCGH was also used to examine the 7q21-q22 region for its involvement in HCC and found alterations in PFTAIRE protein kinase 1 (PFTK1), ODAG, CDK6, CAS1, PEX1, SLC25A, and PEG10 within this region *(109)*. The authors suggest that upregulation of PFTK1, in particular, may confer a motile phenotype in malignant hepatocytes that correlates with metastasis.

Tumor recurrence complicates resection in a large percentage of cases due to either true metastases or development of de novo tumors.

Vascular invasion, multinodularity, and degree of differentiation are the major predictors of recurrence. Kurokawa et al. identified a 20-gene signature using a PCR-based platform that could predict recurrence with 70% accuracy in an independent cohort of 40 patients *(129)*. A cDNA-based study of 18 HCCs found a 14-gene signature that differed between vascular invasion status and could predict post-resection recurrence *(130)*. cDNA array of HCCs identified claudin-10 expression level to be associated with disease recurrence and was validated by qRT-PCR and associated with survival in multivariate Cox regression analysis *(131)*. In addition, cDNA analyses found gene sets linked to early intrahepatic recurrence including a downregulation of immune response-related genes encoding MHC class II antigens (HLA-DRA, HLA-DRB1, HLA-DG, and HLA-DQA) *(132, 133)*. cDNA arrays have also been used to identify a 46-gene signature associated with extrahepatic recurrence *(134)*. Meanwhile, a 12-gene OLIGO array-based signature has also been shown to predict recurrence within 1-year post-surgery with 93% accuracy *(135)*. A recent follow-up study showed that 3 of these 12 genes (HLA-DRA, DDX17, and LAPTM5) could predict early intrahepatic recurrence with 81% accuracy and were independent risk factors associated with recurrence in a multivariate analysis *(136)*. Another OLIGO study identified a 57-gene signature that could predict recurrent disease at diagnosis with 84% accuracy and was validated in an independent test set *(137)*. The 20-miRNA metastasis signature identified in our laboratory was also significantly associated with recurrence in early-stage HCC *(138)*.

Studies have suggested that while tumor cells affect metastatic capacity, the organ microenvironment can also contribute to this phenotype *(139–141)*. To determine the role of the hepatic microenvironment in HCC metastasis, our laboratory compared the cDNA profiles of noncancerous surrounding hepatic tissues ($n = 115$) from HCC patients with venous metastases which we termed a *m*etastasis-*i*nclined *m*icroenvironment (MIM) sample to those without detectable metastases, which we termed a *m*etastasis-*a*verse *m*icroenvironment (MAM) sample *(138)*. We identified a unique change in the gene expression profiles associated with a metastatic phenotype which was refined to 17 immune-related genes. This signature was inherently different from the HCC tumor signature found in our laboratory and was validated in an independent cohort ($n = 95$). The non-tumor signature could successfully predict venous and extrahepatic metastases by follow-up with >92% overall accuracy and was a superior and independent prognostic indicator when compared with other available clinical parameters for determining patient survival or recurrence. Dramatic changes in cytokine responses, favoring an anti-inflammatory microenvironmental condition, occur in MIM samples, where a predominant Th2-like cytokine profile, favoring a humoral response, was associated with MIM

cases. Colony stimulating factor-1 (CSF1) may be one of the cytokines overexpressed in the liver milieu that is responsible for this shift.

Metastasis and recurrence continue to plague HCC patient outcome. Array profiling methods have identified many alterations that occur in HCC metastasis, some involving well-known metastasis-associated factors such as the angiogenesis-related VEGF and others identifying novel players related to this phenotype. In addition, permissive microenvironments have also been shown to influence HCC metastasis. These metastasis signatures have broadened our knowledge of the biological pathways that are affected during this process and have highlighted particular biomarkers that may be useful to identify HCC patients who are prone to metastasis/recurrence and are tools that can be used to stratify patients for adjuvant therapy. However, the signatures discussed above are largely non-overlapping, suggesting a significant heterogeneity. Although some of these markers have been associated with outcome, future validation and functional/mechanistic studies will be needed to assess their prognostic significance.

3.3. Hepatic Stem Cell Signatures

The heterogeneic nature of HCC and variability of its prognosis suggest that this disease may comprise several distinct biological subtypes. As discussed, microarrays have aided in characterizing separate HCC subtypes with distinct molecular features. Differences in HCC subtypes may arise from activation of different oncogenic pathways during tumorigenesis and/or from different cell origins. Microarray analysis can aid in determining the characteristics of separate HCC subtypes that can provide insight into the cellular origin of the tumor.

Recent studies suggest that HCC may arise from liver stem cells or cells with stem cell-like features which are capable of cellular plasticity, dynamic cell motility, and integral interaction with the microenvironment and are associated with poor outcome. Integrated gene expression data from fetal hepatoblasts and adult hepatocytes with HCC from human and mouse models found that individuals with HCC who shared a gene expression pattern with fetal hepatoblasts had a poor prognosis *(142)*. The gene subset included markers of hepatic oval cells, suggesting that HCC of this subtype may arise from hepatic progenitor cells and analyses of gene networks revealed an activation of AP-1 transcription factors. In our laboratory, we have used cDNA arrays to identify a HCC subtype with features of hepatic stem cells that express AFP and a cell surface hepatic stem cell marker EpCAM *(143, 144)*. EpCAM-positive cells from this subtype have self-renewal and differentiation traits and can initiate highly invasive HCC in NOD/SCID mice (Yamashita et al., unpublished data). The Wnt/β-catenin signaling pathway is augmented in this subtype suggesting that therapeutic approaches geared

toward Wnt/β-catenin signaling inhibitors may impact the survival of HCC patients with this stem cell-like subtype. We have also recently found that miRNAs are associated with this stem cell-like HCC subtype, suggesting that targeting miRNA pathways may alleviate the poor prognosis of HCC patients (Ji et al., unpublished data). However, others have shown that HCC cells that are positive for CD133 or CD90 also have features of cancer stem cells *(145, 146)*. Thus, it appears that hepatic cancer stem cells may also be heterogeneous. It has yet to be determined whether such heterogeneity is due to transformation of different types of stem/progenitor cells or de-differentiation of mature cells.

Recent studies have identified stem cell-like/progenitor cell-like subtypes of HCC that are associated with poor outcome. A clear understanding of these HCC subtypes may identify specific factors that determine more aggressive HCC. Biomarkers associated with these subtypes may help to refine treatment options by allowing more sensitive HCC subtype classification. Furthermore, functional/mechanistic follow-up studies of these stem cell-related biomarkers will aid the generation of novel therapeutic approaches to block pathways associated with poor outcome and thus help to alleviate dismal prognosis.

4. CANDIDATE SERUM MOLECULAR MARKERS

The identification and validation of molecular biomarkers, such as those described above, are relevant toward understanding the pathways that are important for HCC-related disease. Several of these HCC biomarkers have also been associated with diagnosis and prognosis. Importantly, some studies have been validated in independent cohorts and include markers that are expressed in sera, paving the way for clinically useful platforms to assess HCC risk and outcome. Some examples of serum biomarkers identified by HCC array studies are presented below.

4.1. Diagnostic Serum Markers

4.1.1. α-FETOPROTEIN (AFP)

Since its detection in the serum of HCC patients in 1970s, AFP has been the only serological marker widely used for diagnosing HCC patients. This marker allows for the identification of a small set of HCC patients at an early stage with smaller tumors who have a relatively long-term survival rate following curative treatment *(9, 15, 147)*. Recent array studies have shown that AFP status not only distinguishes HCC from normal but can also be useful in distinguishing HCC subtypes with differing prognostic outcome *(101, 143, 144)*. Although other diagnostic markers have been tested for

HCC diagnosis, without sufficient sensitivity and specificity AFP remains the only universally accepted HCC biomarker in clinical practice. However, non-specific elevation and differences in AFP status among ethnic groups remain to be addressed.

4.1.2. Glypican-3 (GPC3)

Glypican-3 (GPC3) is a member of the glypican family of glycosylphosphatidylinositol-anchored cell-surface heparan-sulfate proteoglycans that interacts with and modulates various growth factors *(148)*. Recent studies indicate that GPC mRNA levels are increased in a large proportion of HCC *(149)*. The level of GPC3 in serum is significantly higher in patients with HCC when compared to healthy patients and is detectable in 40–53% of patients with HCC and in approximately one-third of patients with HCC with normal AFP levels *(150–152)*. Moreover, the expression of GPC3 is independent of the differentiation status and size of HCC *(152)*. In addition, using a cDNA approach, our laboratory has found that an increased expression of GPC3 is associated with most HCC samples including those with normal serum AFP and small tumor size *(54)*. GPC3 was also shown to be upregulated in HCC using cDNA arrays in an independent study showing a link with integrin and Akt/NF-kB pathways *(64)*. This protein is a promising new diagnostic biomarker for HCC.

4.1.3. Midkine (MDK)

Midkine (MDK) encodes a novel heparin-binding growth factor originally identified in embryonal carcinoma cells that is involved in the early stage of retinoic acid-induced differentiation *(153)*. Analogous to AFP, MDK mRNA is highly expressed during embryogenesis but is undetectable in adult tissues except kidney *(154)*. Serum MDK has been reported to be elevated in patients with various types of carcinomas, but not in normal individuals *(155)*. Similarly, an increased expression of MDK has been reported to be associated with HCC *(156, 157)*. Midkine is thought to be involved in carcinogenesis and tumor progression by promoting vascularization, fibroblast growth, and cell migration while suppressing apoptosis *(158, 159)*. In a study performed in our laboratory, MDK was a candidate serum expressed protein that was associated with HCC, including those with normal serum AFP and small tumors *(54)*. These studies suggest that MDK plays an important role in carcinogenesis and the development and metastasis of tumors and that it could serve as a novel tumor marker. Since MDK can be detected in serum, it may be offered as a potentially less invasive diagnostic marker, especially for those who are negative for AFP. Further studies will be needed to validate its use.

4.1.4. Cystatin B (CSTB)

Cystatins are endogenous inhibitors of lysosomal cysteine proteinases *(160)*. Cystatin B (CSTB) is a member of the cystatin superfamily and mutations resulting in a loss of function are responsible for an inherited, progressive, and lethal autosomal disease *(161)*. Furthermore, the activity of CSTB has been reported in several human carcinomas and is overexpressed preferentially in HCC *(44, 162–164)*. In addition, CSTB protein levels were detectable in HCC tumor tissues compared with corresponding non-tumor tissues, and CSTB level was significantly elevated in HCC serum compared with healthy patients or those with chronic liver disease. Therefore, CSTB is specifically overexpressed in HCC tissues and in HCC patients. Whether other CSTB family members are associated with HCC remains to be elucidated.

4.1.5. Complement C3A (C3A)

Complement (C3a) components are important mediators of inflammation and contribute to the regulation of the immune response. Complement activation with subsequent deposition of complement components on tumor tissue has been observed in cancer patients *(165)*. Human C3a is the most abundant complement protein in serum and has been reported to contribute to the early priming stages of hepatocyte regeneration after toxic injury and partial hepatectomy *(166, 167, 168)*. Using proteomic arrays to search for HCC biomarkers, C3a was found to be downregulated in HBV-related HCC *(169)*. Meanwhile, other protein array studies have shown that C3a is specifically upregulated in patients with chronic hepatitis C and those with HCV–HCC, highlighting a difference between HBV and HCC *(6)*. The expression of C3a in HCC sera was further validated by PS20 chip immunoassay and Western blotting. The level of C3a, however, did not correlate with alanine aminotransferase (ALT) values, tumor size, or cirrhosis in chronic hepatitis C and HCV-related HCC groups. Although C3a did not correlate with known clinical parameters, it may be an independent marker for chronic hepatitis C and HCV-related HCC. Taken together, these findings suggested that C3a is associated with the process that leads to the development of HCC.

4.1.6. Insulin-Like Growth Factor (IGF-II)

Insulin-like growth factor (IGF-II) is a mitogenic polypeptide closely related to insulin that serves as an autocrine growth factor in various cancers and is highly expressed during hepatocarcinogenesis *(170, 171)*. It is also associated with the induction of various angiogenesis factors *(172)*. Two comparative studies of AFP and IGF-II serum levels in HCC patients and cirrhotic or normal control subjects found that these two markers were closely

associated in terms of expression and could function as complementary tumor markers *(173, 174)*. IGF-II was increased in HCC patients as compared to cirrhotic and normal controls. In cDNA array studies of 43 different human HCC samples and 3 HCC cell lines in comparison with normal adult liver, two main groups of HCC (designated group A and group B) were identified *(100)*. Based on the expression pattern, group B was further subdivided into two subgroups. A prominent characteristic of subgroup B1 and HCC cell lines was the overexpression of insulin-like growth factor IGF-II. Moreover, IFN-γ treatment substantially reduced IGF-II expression in HCC cells. In a proteomic array study of 210 HCC specimens and corresponding liver tissue, IGF-II was significantly upregulated in HCC and was confirmed by Western blot analysis and TMAs *(175)*. This profiling may be of mechanistic and therapeutic impact because IGF-II overexpression has been linked to reduced apoptosis and increased proliferation and may be accessible to therapeutic intervention. IGF-II may also play an important role in the development of neovascularization and HCC metastasis and may therefore be a useful marker not only for diagnosis but also for prognosis *(176, 177)*.

4.2. Prognostic Serum Markers

4.2.1. Osteopontin (OPN)

Osteopontin (OPN, SPP1) is a secreted multifunctional glycoprotein expressed at high levels in tumors and the surrounding stroma of numerous cancers, including the liver *(178–180)*. Increased serum and plasma OPN levels are associated with advanced-stage lung, breast, colon, and prostate carcinomas *(181–183)*. Importantly, OPN expression can predict high-grade, late-stage, and early-recurrence HCC and is highly correlated with tumor recurrence and decreased patient survival following orthotropic liver transplantation *(184)*. OPN was also shown to be upregulated in HCC using cDNA arrays in an independent study showing a link with integrin and Akt/NF-kB pathways *(64)*. In our laboratory, we have shown that OPN is a significant factor in HCC metastasis *(122)*. Similar findings have been shown in metastatic tumor cell lines and breast cancer patients *(185–187)*. Furthermore, a neutralizing antibody to OPN can decrease pulmonary metastases in nude mice and inhibit tumor cell invasion, highlighting an essential role of OPN in HCC metastasis *(122)*. We have also found that elevated expression of OPN is concordant with matrix metalloproteinase-9 (MMP-9) in primary metastatic HCC *(188)*. We found that MMP-9 cleaved OPN into specific fragments, one of which (OPN-5kD, residues 167–210) could induce low-metastatic HCC cellular invasion via CD44 receptors which was effectively blocked by the addition of small peptides within the region of OPN-5kD. In addition, increased expression of an OPN splice variant (OPN-c) was

associated with clinical metastatic HCC. Thus, a distinct region of OPN was shown to be most essential for HCC cellular invasion and appears to correlate with their metastatic potential. Our data also suggest an alternative splicing event (OPN-c) promotes extracellular cleavage of OPN by MMP-9 to release OPN-5kD. These findings may help to improve advanced-stage HCC prognosis and suggest the utility of small peptides for novel therapies.

4.2.2. Colony Stimulating Factor-1 (CSF1)

Macrophage colony stimulating factor (CSF1), originally identified as a hematopoietic growth factor, is a dimeric polypeptide growth factor that acts through the cell surface receptor (CSF1R) that stimulates proliferation, differentiation, and survival of monocytes and macrophages *(189)*. CSF1 was originally identified as a regulator of the proliferation, differentiation, and survival of macrophages and their bone marrow progenitors *(190)*. However, in addition to its normal role in mononuclear phagocyte biology, elevated expression of CSF-1 and *cfms* has been found in breast, uterine, and ovarian tumor cells, and the extent of expression in these tumors correlates with high grade and poor prognosis *(191–193)*. The biological role and possible clinical significance of these macrophages are still unknown and remained controversial. Studies have shown that macrophages can serve as both positive and negative mediators of tumor growth. Macrophages are known to mediate direct antitumor cytotoxicity and the presentation of tumor-associated antigens *(194)*. On the other hand, macrophages have also been found to promote tumor angiogenesis and to secrete a wide range of growth factors which may promote tumor growth *(195)*. However, as most of these data are derived from studies of cultured tumor cells or from clinical observations, the functions for macrophages in the tumor microenvironment have still not been determined.

In HCC, we have shown that a unique inflammation/immune response-related signature is associated with noncancerous hepatic tissues from metastatic HCC patients and is principally different from that of the tumor. A global Th1/Th2-like cytokine shift in the venous metastases-associated liver microenvironment coincides with elevated expression of CSF1. A refined 17-gene signature containing CSF1 was validated as a superior predictor of HCC venous metastases in an independent cohort, when compared to other clinical prognostic parameters. Our results show that the T cell population may be involved in the promotion of Th2 cytokines and repression of Th1 cytokines in peripheral blood mononuclear cells (PBMC) induced by CSF1. It is possible that these T cell populations are differentially primed in pro-metastatic conditions, in part by the activity of CSF1, and thus produce cytokine profiles that favor cancer advancement. We suggest that a predominant humoral cytokine profile occurs in the metastatic liver milieu and a

shift toward anti-inflammatory/immune-suppressive responses may promote HCC metastases.

4.2.3. Vascular Endothelial Growth Factor (VEGF)

Angiogenesis is a neovascularization process essential for tumor growth, invasion, and metastasis *(196, 197)*. Angiogenesis is regulated by various angiogenic factors of which vascular endothelial growth factor (VEGF) seems to play a central role *(198)*. The elucidation of the mechanisms of angiogenesis is of importance because anti-angiogenic agents are now available and may be of potential benefit in patients with HCC *(199)*. VEGF overexpression and increased serum level has been associated with a greater risk of metastasis, recurrence, and poor survival in HCC *(200–202)*. VEGF was among the top angiogenic factors expressed in HCV–HCC tissues in an OLIGO array study compared to normal livers *(50)*. Moreover, VEGF was also differentially expressed when HCV–HCC samples were compared to HCV cirrhotic tissues. In a TMA study, it has also been shown that Id-1 (inhibitor of differentiation/DNA synthesis), which belongs to the Id family of helix–loop–helix proteins, might enhance HCC angiogenesis and metastasis through interaction with VEGF *(126)*. Therefore, soluble angiogenic factors, such as Id-1 and VEGF, might be useful for monitoring high-risk HCV patients and might be novel targets to inhibit HCC metastasis through suppression of angiogenesis.

4.2.4. Angiopoietins (Ang-1 and Ang-2)

Angiopoietins (Ang) are endothelial cell growth factors which act as ligands for the tyrosine kinase receptor, Tie2. The Ang-1/Tie-2 pathway is thought to mediate the vital functions of vascular stabilization and vascular remodeling, via integration of periendothelial cells into the vascular wall, particularly in the presence of VEGF. In contrast to Ang-1, Ang-2 induces vascular regression in the absence of VEGF but increases vascular sprouting in its presence *(203)*. Overexpression of Ang-2 has been associated with poor prognosis and reduced disease-free survival in several human cancers, including HCC *(204)*. It has been shown that the ectopic expression of Ang-2 in HCC cells promotes rapid development of tumor and aggravates its prognosis, suggesting that the Ang-2/Tie-2 pathway might be involved in angiogenesis of HCC. Thus, increased expression of Ang-2/Tie-2 appears to play a role in promoting tumor angiogenesis in human HCC *(205, 206)*. In a human angiogenesis OLIGO array, Ang-1 and Ang-2 were overexpressed in HCV–HCC *(50)*. In addition, serum levels of Ang-2 were found to be elevated in patients with cirrhosis and more so in HCC *(205)*. Thus, monitoring the serum level of angiogenic factors may be helpful in clinical recommendations for HCC.

4.2.5. Fibroblast Growth Factor (FGF)

Fibroblast growth factor (FGF) is a soluble heparin-binding polypeptide with a potent mitogenic effect on endothelial cells. The upregulation of FGF has been associated with tumor metastasis and recurrence in HCC *(124)*. In a TMA study, FGF was shown to be elevated in HCV–HCC samples *(50)*. In separate studies, serum FGF-2 was significantly elevated in patients with HCC compared with healthy volunteers and circulating basic FGF plasma levels were an indicator of CLD progression *(207, 208)*. The prognostic significance of serum FGF following resection for HCC was evaluated by Poon and colleagues who found that high levels of FGF independently predicted decreased disease-free survival on multivariate analysis in a series of 88 patients *(209)*. This finding indicates that upregulation of FGF may play an important role in HCC metastasis and recurrence. Further study of FGF may provide a new insight to evaluate HCC metastasis and prognosis.

4.2.6. Hepatocyte Growth Factor (HGF)

Hepatocyte growth factor (HGF) is a multifunctional cytokine that affects mitogenesis, cell motility, matrix invasion, and epithelial carcinogenesis *(210)*. In a human angiogenesis OLIGO array, HGF was found to be overexpressed in HCV–HCC *(50)*. Several reports have shown increased serum HGF levels in patients with chronic hepatitis infection and HCC *(211–214)*. High HGF concentrations were associated with a significantly increased risk of HCC development and some studies have shown an association with tumor metastasis and poor prognosis after hepatic resection *(215–217)*. Hepatocyte growth factor may, therefore, be a target of future HCC postoperative treatment. Additional studies will be needed to determine whether inflammatory changes rather than hepatic carcinogenesis are responsible for increased serum HGF levels in patients with chronic hepatitis and HCC.

4.2.7. Interleukin-6 (IL-6)

Interleukin-6 (IL-6) is cytokine associated with the inflammatory process. Although considered to be hepatoprotective *(218)*, it has also been shown that persistent high levels of IL-6 causes liver damage *(219)*. In an OLIGO-based microarray study of HCV core-infected hepatocytes, IFN-stimulated genes were increased, including IL-6 *(220)*. The authors suggested that IL-6 could play a role in modulating cell growth through alterations in Stat3 signaling and regulation of c-myc and cyclin D. Other studies have shown that IL-6 levels increase upon both HCV infection and expression of HBx *(221, 222)*. The circulating serum level of IL-6 has been associated with many cancer types and was shown to correlate with invasion and metastasis *(223)*. In HCC, higher serum IL-6 was observed in comparison to patients with cirrhosis or normal controls and was significantly more discriminate than AFP *(224)*. In a study of 80 HCC patients, however, IL-6 serum levels

did not correlate with outcome *(225)*. Kupffer cells, the liver macrophages, express IL-6; however, various human tumor cells can produce IL-6 and thus affect disease severity *(226)*. Since IL-6 is involved in HCC progression, this cytokine may be useful as both a diagnostic and a prognostic marker. Further studies will be needed to validate these findings.

Thus, several serum-based biomarkers have been identified from array-based studies. Interestingly, biomarkers associated with inflammation and angiogenesis have been predominantly found to be associated with HCC prognosis, reinforcing the importance of changes in the immune system and phenotypes of metastasis on patient outcome. AFP, however, still remains the most sensitive and specific biomarker for HCC diagnosis and prognosis. Improvements in measurement and perhaps combinatorial studies will provide more sensitive/specific biomarkers in the future. These examples of diagnostic and prognostic serum markers, however, are notable advances in the application of information gained from array-based studies toward clinical practice.

5. SUMMARY

The advent of microarray technology has provided a high-throughput methodology to assess the genome-wide changes that occur during hepatocarcinogenesis. Using multiple sample types, array platforms, and data analysis methods, the mechanisms related to HCC carcinogenesis can be elucidated and related to disease pathogenesis and clinical measures. The definition of molecular markers from these studies has the potential to revolutionize the diagnosis and prognosis of patients with HCC.

Microarrays have steadily become more comprehensive and stable, not only increasing the number of elements that can be arrayed but also expanding with regard to the types of material that can be analyzed. Despite advances in stability and composition of microarrays, several fundamental issues still remain to be resolved. These include multiple sources of variation (among samples, within arrays, mixed cell types, user-related error, etc.) which may lead to overinterpretation or spurious functional gene associations. In addition, the need for physical destruction of cells/tissues limits consequential assays conducted on the same material. Advanced techniques such as laser capture microdissection and automation have somewhat improved these challenges. The overall quality and amount of starting material is a major challenge and is limited by the amount and complexity of the sample as well as user-related handling. In addition, many oncogenic processes are not accounted for by array analysis since they are regulated post-transcriptionally. Therefore, elements such as protein localization and modification need to be included in HCC profiling. Difficulties in data comparison must also be addressed which ensues from the use of multiple array

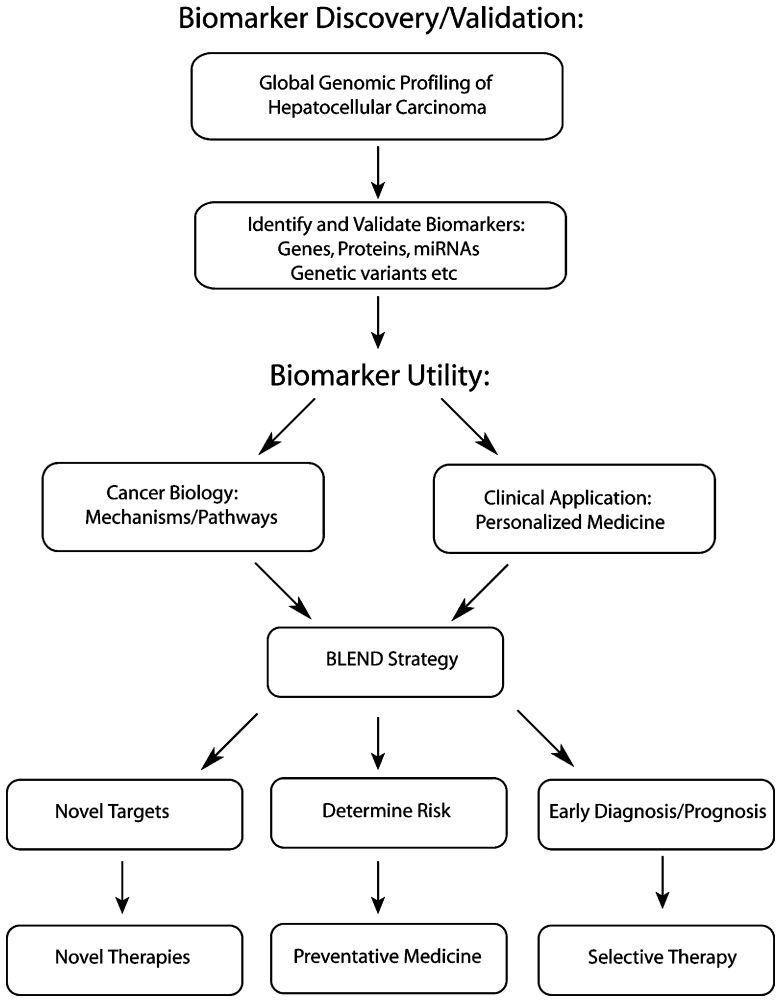

Fig. 1. Global expression-based biomarker identification, validation, and clinical utility. Wide-screen genomic profiling of hepatocellular carcinoma (HCC) has identified multiple biomarkers on the gene, protein, and genomic scale. These biomarkers are useful for understanding HCC biology and clinical application. The mechanistic and clinical information gleaned from genomic profiling studies can be combined using a Biological Expression Network Discovery(BLEND) strategy to identify promising novel therapeutic markers for diagnosis, treatment, and prognosis of HCC. Such methods will allow progression toward personalized medicine encompassing new and selective therapeutics and preventative therapy.

platforms and data algorithms among published studies as well as frequent updates of genomic databases. Such problems may be alleviated by setting adherence guidelines for microarray statistical analysis and reporting such

as those established by the International Microarrays Gene Expression Data group, the REMARK guidelines, or incorporation of proper study design that is suitable for array-based biostatistical analyses *(227–229)*. Resolution range is a large limitation in array analysis, whereby important changes may not be assessed or studied due to the cutoff criteria in the analysis. In addition, each microarray can only provide information concerning the targets that are included on that array. Future studies may require integrative analysis of multiple platforms in order to define the exact cancer-related molecular changes on multiple biological levels and to distinguish the key players from their downstream effects. Advancement in statistical methods to integrate multiple platforms will be required to make such assessments. Recently, systems have been developed (e.g., Illumina Genome Analyzer) that offer whole-genome analysis using a massive parallel sequencing that is useful for discoveries in genomics, epigenomics, gene expression, and protein–nucleic acid studies. Such systems offer an extremely high-throughput method to complete large-scale global studies in an accurate manner and may allow for ease in cross-platform-type analyses since an enormous multilevel data set can be achieved with a relatively small amount of the same starting material. The utilization of a Biological Expression Network Discovery (BLEND) strategy integrating global molecular profiling data along with mechanistic/functional studies may improve the diagnosis, treatment, and prognosis of HCC patients (Fig. 1).

Although multiple publications have identified and validated diagnostic and/or prognostic HCC markers (Table 2), critical challenges in translating the findings to clinical practice remain. To reach clinical applicability, the measurement of biomarkers must be reproducible, reliable, and easily accessible by non-invasive methods. In addition, the biomarker sets will need to be refined to a smaller number of informative biomarkers to be useful for clinical interrogation. Large prospective studies will need to be performed to assess appropriate sample size for accurate diagnostics and appropriate validation cohorts will be needed to incorporate gender, race, and underlying etiological differences among HCC patients. Nonetheless, the biomarkers that have been identified through gene profiling, particularly those expressed in serum, are an unprecedented advance toward useful clinical application.

Overall, molecular profiling studies have become powerful methods to incorporate global genomic readouts with biological effects and are conduits for the discovery of biomarkers with potential clinical application. The HCC-related genomic expression studies presented in this chapter along with future studies and advances in microarray technology, experimental design, and statistical analyses will undoubtedly lead to crucial and important progress in our understanding of the molecular mechanisms and biology of HCC. Moreover, these studies have revealed molecular markers that provide the framework toward predictive and personalized care for HCC

Table 2
HCC Clinical Markers Defined by HCC Microarray

Diagnosis/prognosis		*Platform*	*Prediction signatures*	*Top five significant genes/miRNAs/proteins*	*Ref*
Diagnosis	**Early HCC**	**cDNA**	25 genes	Not listed	*(44)*
			40 genes	Not listed	*(104)*
		Proteome	6 proteins	DEAD box polypeptide 3, eEF2, AIF, hnRNP A2, prostatic-binding protein, TIM	*(242)*
	HCC subtypes	**cDNA**	83 genes	*ACP5, RPL39L, TACSTD1, H1F0, H19* in HBV-HCC; *C1S, IFI27, TAT, AZGP1* in HCV-HCC	*(45)*
			59 genes	Not listed	*(62)*
			HCC subtype genes	Not listed	*(100)*
			2 genes	*EpCAM, AFP*	*(144)*
	Staging	**cDNA**	240 genes	Not listed	*(105)*
			31 genes	*IQGAP1, PPT1, CTSC, LIPA, SNX2*	*(103)*
	HCC markers	**cDNA**	50 genes	*PGCP, PLA2G13, and PLA2G7*	*(58)*

Table 2 (Continued)

Diagnosis/prognosis		*Platform*	*Prediction signatures*	*Top five significant genes/miRNAs/proteins*	*Ref*
			68 genes	Not listed	*(61)*
			44 genes	Not listed	*(59)*
			63 genes	Not listed	*(102)*
			217 genes	Not listed	*(115)*
			5 genes	*GPC3, PEG10, MDK, SERPINI1, QP-C*	*(54)*
			248 genes	*EIF3S3, C9, UBD, FLJ42752 fis, SLC12A8*	*(239)*
		microRNA	8 miRNAs	miR-18, miR-199a∗, miR-199a, miR-224, miR-195	*(89)*
			40 miRNAs	Not listed	*(241)*
		Proteome	250 features	Not listed	*(97)*
			4 peptides	7486, 12843, 44293, 53598 Da	*(99)*
			11 peaks	Cystatin C	*(98)*
Prognosis	**Metastasis**	**cDNA**	90 clones	Not listed	*(114)*
			30 genes	Not listed	*(122)*
			35 genes	*HFL3, PMS2L11, SGK, GPRK6, ZNF216*	*(238)*
			17 genes	Not listed	*(138)*

(*Continued*)

Table 2
(Continued)

Diagnosis/prognosis	*Platform*	*Prediction signatures*	*Top five significant genes/miRNAs/proteins*	*Ref*
	microRNA	20 miRNAs	miR-30c-1, miR-1-2, miR-34a, miR-148a, miR-124a-2	*(123)*
Recurrence	**cDNA**	3 genes	*HLA-DRA, DDX17, LAPTM5*	*(135, 136)*
		20 genes	*ALCAM, FLJ37965, NRG2, CDH1, RGS5*	*(129)*
		4 genes	*HLA-DRA, HLA-DRB1, HLA-DG, HLA-DQA*	*(235)*
		36 genes	*TRIM25*, *EIF2S3*, *CLECSF14*, *DXYS155E*	*(60)*
		14 genes	Not listed	*(130)*
		46 genes	*MPV17, GZMB, ITGA6*	*(134)*
		57 genes	*USH1C, CNGA1, INSIG1, RACGAP, GSTM3*	*(137)*
HCC risk	**cDNA**	30 genes	*CP, CACNB3, MT1E, DYRK3, UAP1*	*(90)*
Markers	**Genome**	2 regions	Loss on 17p13.3; Gain on 8q11	*(81)*
	cDNA	406 genes	Not listed	*(119)*
		Angiogenesis soluble factors	Not listed	*(50)*

patients. We are now at the brink of clinically implementing biomarkers identified from global gene expression profiling to improve HCC diagnosis, treatment, and outcome.

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6 Pathologic Aspects of Hepatocellular Tumors

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CONTENTS

ABSTRACT

Hepatocellular tumors are pathologically divided into a limited number of entities such as focal nodular hyperplasia, hepatocellular adenoma, hepatocellular carcinoma and its variants, and hepatoblastoma. Recent advances in immunophenotypic and molecular characterization have led to an increased appreciation of the complexities of these growths. For example, subtypes of hepatocellular adenomas with differing premalignant potentials have been defined, our ability to differentiate hepatocellular carcinoma from high-grade dysplasia continues to improve, and molecular similarities of histologically discordant elements of combined hepatocellular/cholangiocellular carcinoma have been reported. This chapter describes

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pathologic, immunophenotypic, and molecular features of hepatocellular tumors. Continued progress in our understanding of these growths at the cellular and subcellular levels suggests that categorization of these tumors may continue to evolve as additional significant clinicopathologic correlates are discovered.

Key Words: Focal nodular hyperplasia; hepatocellular adenoma; hepatocellular carcinoma; dysplasia; hepatoblastoma; histopathology; immunophenotypic analysis; molecular pathology; tumor staging

1. INTRODUCTION

Hepatocellular tumors are conveniently divided into a limited number of pathologic categories in order to provide a simplified framework allowing rational application of diagnostic and therapeutic procedures. However, such an approach understates the tremendous range of cellular and architectural variation of these tumors, attributable to the wide plasticity of the hepatocyte and its progenitors. This chapter categorizes hepatocellular neoplasias and relevant non-neoplastic growths using established pathologic headings. The ongoing application of molecular techniques to enhance and sometimes transform our understanding of these lesions provides a recurrent theme throughout the discussion. In addition to comprehending the accepted relationships among the various tumors, the reader is challenged to consider alternative relationships that may conceivably mirror the underlying biology in a more accurate fashion. Such examples might involve the presence of mesenchymal metaplasia in lesions as seemingly diverse as hepatoblastoma and mixed hepatocellular carcinoma/cholangiocarcinoma. One may also ask if specific molecular pathways such as β-catenin/Wnt or specific cell types such as bipotential progenitor cells may define subsets of similar tumors that cut across current established morphology-based classifications.

2. FOCAL NODULAR HYPERPLASIA

2.1. Clinical Aspects

Focal nodular hyperplasia (FNH) is a benign mass lesion that arises from a hyperplastic response to locally malformed vasculature and resultant increase in regional blood flow *(1–3)*. FNH can occur in either sex and at any age, although it is most common in women of reproductive age. Estrogen use is not considered to be directly causative but may be associated with lesion growth *(4)*. Rapid growth of FNH in the absence of estrogen use has also been reported *(5)*.

FNH has been associated with other conditions characterized by local vasoformative anomalies such as hepatic hemangiomas or hereditary hemorrhagic telangiectasia *(6)*. Increased frequency of FNH has been reported after anti-neoplastic therapy, where it has been suggested that the increase may relate to vascular injury associated with such treatment *(7)*.

The radiographic appearance of typical FNH is diagnostic and most cases are detected incidentally during abdominal radiographic examination for other conditions. Occasionally it may present as fullness or a mass lesion.

FNH is usually a clinically benign condition and in many cases it can be followed without surgical intervention. Rarely, larger lesions may undergo significant hemorrhage *(8)* and exceptionally, hepatocellular carcinoma has been observed to arise within these hyperplasias *(9)*.

2.2. FNH Macroscopic Aspects

FNH presents as a discrete unencapsulated mass lesion with a lobulated appearance accentuated by bands of fibrosis. These fibrous septa typically radiate from the center of the lesion, where they coalesce into a larger central scar (Fig. 1). This characteristic feature facilitates radiographic diagnosis in most cases. Variations include eccentric scars and multiple smaller fibrous scars. Importantly from a diagnostic perspective, hepatocellular carcinoma may on occasion also contain a central scar and must be distinguished from FNH *(10)*.

A dystrophic vasculature is a ubiquitous feature of FNH and this may be macroscopically detectable in some cases as isolated and enlarged vessels within or at the periphery of the growth. In the recent past, some liver

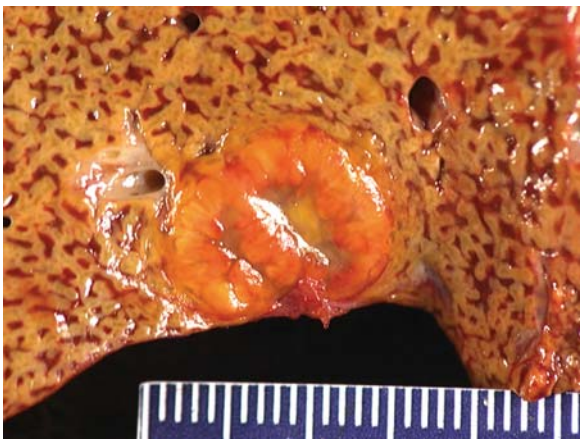

Fig. 1. Focal nodular hyperplasia arising in a noncirrhotic liver. The nodule has centrally depressed areas corresponding to the central fibrous scar. The background liver shows chronic passive congestion.

masses characterized by an excess of vasculature with minimal fibrosis were referred to as telangiectatic FNH; however, clonal studies have unambiguously redefined these tumors as variants of hepatocellular adenomas, and they are discussed in that section (below).

Many but not all FNH are solitary and small. In a recent series, 80% of FNH was under 5 cm, 18% between 5 and 10 cm, and 2% greater than 10 cm in diameter *(11)*. In approximately 20% of cases, multiple FNH coexist. A diagnosis of FNH in one lesion does not ensure that all other lesions are identical, as concurrent hepatocellular carcinoma may also occur in livers harboring FNH *(12, 13)*.

2.3. FNH Microscopic Aspects

The microscopic appearance of FNH is dominated by bland cytology with architectural distortion produced by the central area of fibrosis from which radiate individual fibrous septa that circumscribe complete and incomplete nodules of normal-appearing hepatocytes. When the entire lesion is resected it is not difficult to delineate FNH from the surrounding parenchyma despite both the absence of a pseudocapsule and the bland appearance of hepatocytes.

The fibrous septa contain the dystrophic artery branches that supply the lesion (Fig. 2). These vessels are characterized by asymmetric-appearing muscular layers due to irregularly distributed but benign-appearing areas of muscular hyperplasia throughout their lengths. The recognition of these vessels is of diagnostic importance. Of similar diagnostic import is the

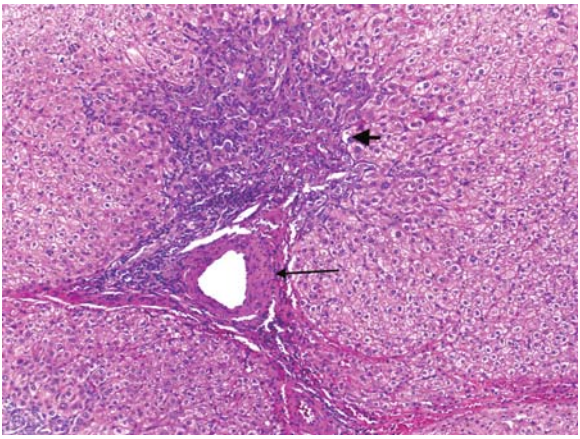

Fig. 2. Focal nodular hyperplasia. A thick walled dystrophic vessel (*thin arrow*) is present within a fibrous area that corresponds to part of the fibrous scar of the lesion. True bile ducts are not present. However, focal cholangiolar proliferation at the interface between fibrous septa and hepatocyte areas may be focally prolific (*thick arrow*) (100×).

absence of accompanying bile ducts in the vicinity of artery branches. On occasion a portal tract may be enveloped within an area of the lesion, but for the most part bile ducts are absent from FNH. In contrast, bile ductular overgrowth is common at the interface between fibrous bands and hepatocyte trabeculae. This may be prolific in some areas and absent in others (Fig. 2), possibly related to microenvironmental differences in blood and bile flow within the lesion. The change is similar to the so-called "biliary interface hepatitis" that occurs with biliary outflow compromise. This similarity extends to the fact that hepatocytes in this area may be swollen due to retained bile salts (cholate stasis). Further, localized increase in copper (and copper binding protein) may occur here and is diagnostically useful as a point in favor of the diagnosis of FNH over other lesions such as hepatocellular adenoma. We have seen rare examples of the latter condition (as well as HCC) producing a positive copper stain, however, and emphasize that the diagnosis must take the entire appearance of the lesion into account.

A needle biopsy may be performed in cases in which the diagnosis is ambiguous by radiographic examination. Several pitfalls may arise in this circumstance. First, if the fibrosis is heavily sampled, a diagnosis of cirrhosis may be entertained. This error may be compounded by the presence of ductular proliferation, in which a biliary etiology might be suggested. Knowledge of the presence of a mass lesion is helpful, and a search for true bile ducts adjacent to artery branches will demonstrate that normal portal tracts are absent. This task can be difficult if some areas do show true ducts. In that case the likelihood that both normal and abnormal areas of liver have been sampled should be considered and an effort to mentally separate these regions undertaken. Examination of the vessels themselves may disclose dystrophic change in some but not other areas and this is a helpful finding.

With knowledge that the biopsy has been performed for diagnosis of a hepatic mass, the differential diagnosis of hepatocellular adenoma often arises, particularly in cases in which ductular proliferation is absent. We find ancillary cytokeratin staining for ductules to occasionally be helpful. In this regard we consider cytokeratin 19 to be more useful than cytokeratin 7, since the latter can often be expressed by hepatocytes adjacent to fibrous regions. Copper stain and search for dystrophic vessels may also be of benefit. Hepatocellular adenomas appear to show a more diffuse distribution of vasculature throughout the hepatocyte regions in contrast to FNH in which the vessels diminish in number and caliber as one leaves the fibrous regions, and occasionally this feature is marked enough to be useful.

It is not always possible to histologically distinguish FNH from hepatocellular adenoma. In some cases clonal or other molecular studies (below) may be of benefit. In other cases clinical circumstances may ultimately dictate whether the lesion is followed by repeat radiographic studies or resected.

3. HEPATOCELLULAR ADENOMA

3.1. Clinical Aspects

Hepatocellular adenoma (HCA) is an uncommon and benign liver tumor arising most frequently in women of childbearing age and with a history of oral contraceptive use. In one early study *(14)*, HCA occurred at a rate of 0.1 per 100,000 women per year when there was no history of oral contraceptives and this rose to 3.4 per 100,000 per year with long-term use of these agents. More recent low-dose formulations do not appear to be associated with this high level of risk. Anabolic steroid use is also associated with hepatocellular adenoma, and an example of this lesion arising in conjunction with growth hormone therapy for Turner's syndrome has been reported *(15)*. Use of the antiseizure medication oxcarbazepine has been associated with HCA in mice and in a single recent clinical case report *(16)*. An association of liver cell adenoma and various genetic metabolic disorders such as glycogen storage diseases types I, III, or IV, galactosemia, and tyrosinemia have been reported. Maturity-onset diabetes of the young, type III (MODY III) and familial adenomatous polyposis are two additional predisposing conditions that have a special relationship with molecular alterations present in HCA and these are considered below.

Many cases are first detected during abdominal scan *(17)* for low-grade symptoms, feeling of fullness, or other conditions. Intratumoral hemorrhage or rupture with hemoperitoneum may occur, particularly with larger tumors. However, in the series of Toso et al. *(18)*, rupture was seen in HCA as small as 1.7 cm, and these authors recommended resection of all HCA insofar as possible. Immediate management of hemorrhage with or without surgery *(19)* and observation of HCA less than 5 cm in size *(20)* have been emphasized by others and the possibility that HCA may regress if hormonal stimulation is withdrawn has also been noted *(21)*. Malignant transformation is an additional known complication of HCA, and Toso et al. *(18)* documented foci of HCC in 8% of their resected HCA.

3.2. Macroscopic Pathology

Hepatocellular adenoma characteristically appears as a well-circumscribed, nonlobulated lesion within a noncirrhotic liver (Fig. 3). Adenomas can range from 1 to over 30 cm but most are between 5 and 15 cm in diameter. Typically adenomas occur in subcapsular locations and in the right lobe. The tumor may be pedunculated *(22)*. It is usually solitary, but multiple lesions can occasionally be seen, particularly in glycogen storage disease type I and in liver adenomatosis *(23–26)*. The latter by definition consists of 10 or more individual adenomas. An association of adenomatosis with hepatic steatosis has been suggested *(27)*.

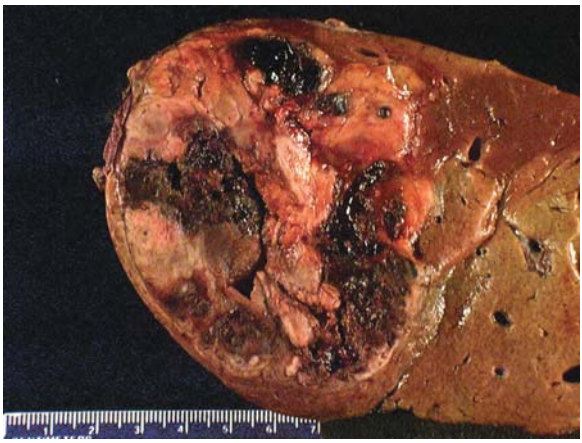

Fig. 3. Hepatocellular adenoma arising in a noncirrhotic liver. This 9.5 cm tumor arose in the noncirrhotic liver of a middle-aged woman with a long history of oral contraceptive use. The dark areas represent hemorrhage that caused pain and led to the discovery of this benign tumor.

Hepatocellular adenomas vary in color from yellow to tan and can be variegated due to a combination of intratumoral hemorrhage, infarction, and fatty changes *(24, 28)*. The tumors are usually unencapsulated.

3.3. Microscopic Pathology

Hepatocellular adenomas contain normal-appearing hepatocytes arranged in a trabecular architecture ranging from one to three cells thick (Fig. 4). There are no portal tracts and therefore the normal hepatic microanatomical relationships are lacking. The hepatocyte nuclei are small, round, and uniform. Nucleoli are inconspicuous. Mitoses are absent or few. Cytoplasm is pale or eosinophilic and marked steatosis may be present. Cholestasis is not uncommon. The normal reticulin pattern is well preserved and Kupffer cells exist in their usual locations. An inflammatory component may be present. Small venous and arterial branches are seen throughout the tumor (Fig. 4). Occasional larger vessels are seen and may also appear as "feeding" vessels adjacent to the tumor. Occasionally the tumoral hepatocytes may contain PAS-positive, diastase-resistant hyaline globules *(29, 30)*, Mallory's hyaline *(31)*, or degenerate-appearing hyperchromatic nuclei *(32)*.

The recent Bordeaux update of liver cell adenoma classification *(1)* has altered our understanding of this lesion and is considered in the next section.

Distinction of hepatocellular adenoma from well-differentiated hepatocellular carcinoma may be difficult or impossible by conventional light microscopy. The clinical context is important in this regard, and the diagnosis of hepatic adenoma outside of the setting of a young woman taking oral

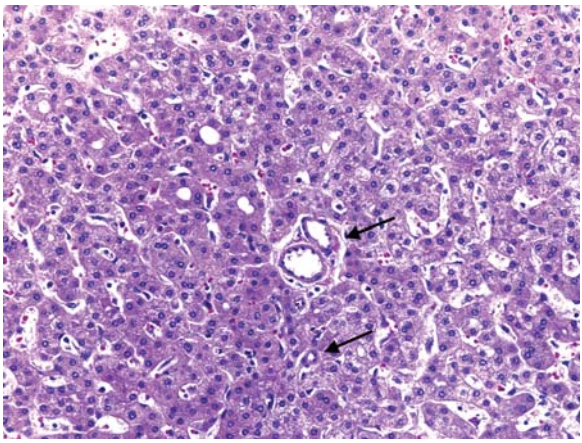

Fig. 4. Hepatocellular adenoma. The tumor is comprised of normal-appearing hepatocytes in an unremarkable trabecular architecture. Isolated artery branches (*arrows*) in the absence of portal tracts do not occur in normal lobules and are consistent with the diagnosis of this lesion as a hepatocellular adenoma (200×).

contraceptives should be viewed with suspicion. Investigations should focus on suspicious-looking areas that are characterized by a clonal appearance (referring to a focus of cells that has a distinctly different look from the surrounding adenoma). This may be due to cytologic differences or to architectural differences such as solid growth or formation of pseudoacini. Micchelli et al. *(33)* extended the earlier finding of Tao et al. *(32)* and noted cytologic atypia as a background change in two of three hepatocellular adenomas harboring foci of hepatocellular carcinoma. This change, demonstrated as enlarged and somewhat hyperchromatic nuclei with underlying intact reticulin architecture, was suggested as a potential risk factor for so-called malignant degeneration of hepatocellular adenoma. However, background atypia was also observed in several other adenomas in which a malignant component was not demonstrated, and the authors concluded that additional studies were necessary to confirm this possible association.

Immunohistochemical and molecular studies are valuable in further characterizing these lesions and are considered next.

3.4. Hepatocellular Adenoma Subtypes and Ancillary Studies

The diagnostic approach to hepatocellular adenomas has been transformed by correlative genotypic and phenotypic studies *(1, 34)* that have led to the recognition of four subgroups with varying risks for malignant transformation. The largest subgroup, comprising between 40 and 50% of adenomas, contains inactivating mutations of the HNF1 alpha gene. In about 85% of cases both mutations are somatic in origin, and in the remaining 15%

one germline and one somatic mutation coexist. Within this latter group are included patients with maturity-onset diabetes of the young type III and a number of patients with a family history of liver adenomatosis. HCA with this mutation characteristically contains significant steatosis and does not show evidence of anaplasia or significant inflammation. Association with hepatocellular carcinoma is estimated at 7% at present. Immunohistochemical absence of liver fatty acid binding protein was associated with this mutation in one study *(34)*.

A minority of HCA, estimated at less than 10%, contains mutations affecting the β-catenin gene. This can be indirectly detected by immunohistochemical demonstration of nuclear translocation of β-catenin. In addition, the products of target genes activated by β-catenin, such as glutamine synthetase, can also be detected *(34)*. These HCA do not usually show the steatosis associated with HNF1α-related tumors but are more likely to contain cellular atypia. These occur more frequently in males, and the association with HCC has been estimated to be approximately 46%.

The remaining HCA do not contain evidence of mutations in either of these genes and likely comprise a heterogeneous group. At present these are subdivided into two categories based on the presence or absence of inflammatory infiltrate. Those with inflammation correspond in part to the previously misnamed telangiectatic focal nodular hyperplasia, now preferably referred to as inflammatory adenoma or telangiectatic adenoma. These lesions have not yet been associated with progression to carcinoma. Immunohistochemical positivity for serum amyloid A2 protein has been suggested as a marker for this variant *(34)*. The second subgroup is comprised of those adenomas without known mutations and without significant inflammation. The association with risk for HCC has been estimated to be approximately 13%.

Demonstration of alpha-fetoprotein positivity is strong evidence in support of hepatocellular carcinoma over adenoma. In our experience, foci of carcinoma may show increased cell cycle activity, highlighted by the proliferation marker Ki-67, in comparison to adjacent adenoma and surrounding liver. Such changes must be interpreted in the context of the overall lesion, i.e., the pathologist must make the interpretation as to whether he or she believes that carcinoma, if found, involves the entire lesion or only a portion of the tumor. Glypican-3 expression favors the diagnosis of hepatocellular carcinoma, as it has not been reported to be expressed in adenomas in several small series *(35, 36)*. Absence of staining does not exclude the possibility of HCC, since the antigen is preferentially expressed on less well-differentiated neoplasms and in one study it was expressed in only 50% of well-differentiated HCC *(35)*. A diffuse, rather than focal, expression of CD34 in tumor-associated vessels is said to favor hepatocellular carcinoma over adenoma *(35)*.

Other immunostains do not add appreciably to the diagnostic information. Estrogen, progesterone, and androgenic steroid receptors have been detected in 26–73% of adenomas in different series *(37, 38)* and may also be seen in hepatocellular carcinoma. Hepatic progenitor cells are identifiable by immunohistochemical means in a considerable proportion of hepatocellular adenomas and support the hypothesis that such cells play a role in the development of hepatic tumors *(39, 20)*. However, their identification does not distinguish benign from malignant tumors.

Comparative genomic hybridization has been suggested as a useful ancillary technique for the distinction of adenoma and carcinoma. Gains and losses of chromosome sites on 1q, 4q, 8p, 8q, 16p, and 17p were found to be the six most frequent alterations in HCC by this approach and detection of one or more of these has been proposed as evidence in support of the diagnosis of carcinoma *(21)*. These authors have updated this technique by utilizing fluorescent in situ hybridization (FISH) to detect quantitative anomalies of chromosomes 1, 6, 7, and 8, thereby distinguishing hepatocellular carcinomas from adenomas and other benign lesions in paraffin-embedded material *(22)*.

Differentiation of hepatocellular adenoma from focal nodular hyperplasia (FNH) also has clinical significance, as FNH is a benign condition that does not have the predisposition to hemorrhage that exists in adenoma, allowing in some cases for a more conservative approach to management *(40)*. (However, it should be noted that rare cases of FNH rupture *(8, 40)* and of hepatocellular carcinoma arising within FNH *(9)* have been recorded.) Magnetic resonance imaging, enhanced CT, scintigraphic findings, and angiography show large peripheral vessels with centripetal flow and are diagnostically useful, but the best method for the differentiation of HA and FNH is surgical biopsy *(41–43)*.

Both FNH and hepatocellular adenoma contain benign-appearing hepatocytes. The presence of fibrous bands with artery branches and peripheral ductular hepatocytes in the absence of true bile ducts is characteristic of FNH. Small vessels are also seen in the lobular portion of FNH, but these derive from the core arteries in the fibrous septa and rapidly diminish in caliber as the distance from the fibrous bands increases. Such a gradient may or may not be apparent in individual adenomas.

4. HEPATOCELLULAR DYSPLASIA

Hepatocellular dysplasia was formally defined by a panel of the International Working Part on the Terminology of Chronic Hepatitis, Hepatic Allograft Rejection, and Nodular Lesions of the Liver in 1995. Lesions were subdivided into dysplastic foci (<1 mm diameter) and dysplastic nodules (≥1 mm diameter) and defined using histologic criteria. These

included variations in nuclear and/or cytoplasmic constituents such that a recognizable cell subpopulation could be distinguished from the surrounding hepatocyte parenchyma. Examples of nuclear changes included alterations in size, at least mild irregularity of nuclear contours, and occasional mitoses. Cytoplasmic changes included basophilia, clear cell change, variation in fat, glycogen, Mallory bodies, or resistance to iron accumulation, any of these features differing from surrounding parenchyma. The net result was often a clone-like population of distinguishable cells with altered nuclear:cytoplasmic ratio. This encompassed a spectrum from mild to severe change, which was arbitrarily divided into low-grade and high-grade forms. The authors realized the inherent difficulty in such an approach and observed that definitive classification, as well as distinction from early HCC, awaited the development of more discriminatory molecular diagnostic tools.

Dysplastic foci have also been subdivided on the basis of cell size into small and large cell types. Large hepatocytes with nuclear variability and prominent nucleoli have been subsequently shown to have a low rate of replication and express p16, prompting the suggestion that it be referred to as large cell "change" rather than dysplasia. In contrast, the small cell variant tends to show a higher proliferative rate than surrounding parenchyma and in one study showed chromosomal changes similar to those of nearby HCC.

4.1. Differential Diagnosis of Hepatocellular Dysplasia

Although our understanding of hepatocellular dysplasia is incomplete, it remains a practical necessity to differentiate these lesions from regenerative nodules at one extreme and hepatocellular carcinoma at the other.

The distinguishing feature of dysplasia is that it leads to the formation of an area in which the hepatocytes differ in a qualitative and/or quantitative fashion from the surrounding parenchyma. Some variables that may lead to this difference are given above. In contrast, regenerative nodules are comprised of normal-appearing hepatocytes and are more likely to contain portal tracts within their substance, without evidence of an aberrant arterial vasculature.

The absence of stromal invasion, which refers to the presence of abnormal hepatocytes directly abutting (without evidence of ductular change) or within portal stroma, has been considered to be the most helpful histologic feature separating dysplasia from HCC, which may exhibit this change.

Di Tommaso et al. *(44)* have recently described the utility of immunohistochemistry in separating hepatocellular dysplasia from early hepatocellular carcinoma. Using an antibody panel consisting of glypican-3, glutamine synthetase, and heat shock protein 70, they found that positivity for any two antibodies yielded a 72% sensitivity and 100% specificity for the diagnosis

of HCC over high-grade dysplasia. All cases of regenerative nodules and low-grade dysplastic nodules were negative for these antibodies. Reference should be made to their illustrations to correctly interpret the qualitative aspects of antibody patterns before applying this to clinical material.

Llovet et al. *(45)* used quantitative real-time RT-PCR to evaluate transcription levels of 55 candidate genes in dysplastic nodules and early hepatocellular carcinomas in patients with underlying hepatitis C virus-associated cirrhosis. They identified a three-gene subset comprised of glypican-3, survivin, and the hyaluronan receptor LYVE-1 that had 95% sensitivity and 94% specificity in distinguishing these two conditions.

5. HEPATOCELLULAR CARCINOMA

5.1. Clinicopathologic Comments

The clinical aspects of hepatocellular carcinoma are dealt with in detail throughout this book and are not repeated here. While the following discussion of hepatocellular carcinoma considers the tumor as a discrete entity, it is emphasized that each HCC likely represents the end result of a number of distinctive and partially overlapping malfunctions of a variety of cellular pathways. Thus, clinically similar HCCs arising in cirrhotic livers caused by alcohol versus infection with hepatitis B or C viruses have likely followed a somewhat different pathogenesis from each other, in addition to differing from HCC arising from a pre-existent hepatocellular adenoma in a noncirrhotic liver of a patient with a history of contraceptive pill use.

Further, we are in a transition period in which progress in molecular analysis is redefining our understanding of disease processes in a stochastic manner. Thus, time-worn descriptive terminology slowly gives way to evolving tumor subclassifications based on distinctive sets of molecular alterations. Which clinicopathologic concepts survive and which are discarded remains to be determined. The two approaches are presented in parallel so that the reader may have an overview of these complementary approaches to tumor pathology.

5.2. Macroscopic Pathology

The majority of hepatocellular carcinomas arise in cirrhotic livers and most frequently involve the right lobe (Fig. 5). The tumors are typically soft, vary in color from gray-green-yellow to light brown, are occasionally bile-stained, and often contain foci of hemorrhage or necrosis. Rarely they may contain a central scar that may mimic focal nodular hyperplasia *(10)*. The tumors can be single or multiple and range from less than 1 cm to over 30 cm in diameter with a tendency toward larger sizes when involving noncirrhotic livers *(46)*.

Fig. 5. Hepatocellular carcinoma (mixed hepatocellular carcinoma and cholangiocarcinoma) arising in a cirrhotic liver. The large and small nodules throughout this liver are consistent with cirrhosis. A hepatocellular carcinoma (*arrow*) is larger and has a different color from the nodules due to bile production. A second white nodule immediately to the left and of similar size was largely necrotic. The small white nodule situated superior to the two larger nodules had features of cholangiocarcinoma. This likely represents a mixed tumor, although molecular analysis was not performed at that time.

A wide variety of macroscopic patterns of tumor growth exist, but these have few clinical correlates. The traditional classification of Eggel *(47)* distinguishes three patterns of hepatocellular carcinomas: multinodular, massive, and diffuse. Multinodular HCC was the most common type in one series. In this pattern multiple tumor nodules are scattered throughout the liver *(46, 48)*. Multinodular HCC is typically associated with cirrhosis *(46)*. In the massive pattern a solitary tumor mass occupies much of the liver and may be associated with smaller satellite nodules. This pattern has been associated with noncirrhotic livers *(46)*. The diffuse pattern is the least common and is characterized by numerous widespread small nodules that mimic cirrhotic nodules; these may virtually replace the liver. In cirrhosis, clinically advanced liver disease has been associated with the diffuse or multinodular patterns of HCC *(48, 49)*. Rarely, HCC may be pedunculated, presumably reflecting an origin within an accessory lobe *(50)*. In one study it was concluded that pedunculated HCC has an unfavorable prognosis if appropriate surgical procedures are not performed during the early stages of development *(51)*.

In more recent macroscopic classifications, hepatocellular carcinomas are further subdivided into two main patterns based on growth characteristics: Expanding or expansive tumors have distinct borders that push aside the adjacent liver, and spreading or infiltrative tumors have poorly defined borders that microscopically invade the adjacent liver *(52, 53)*. Kojiro et al.

(54) applied the terms "distinctly nodular" and "indistinctly nodular" to refer to these growth patterns in small tumors. Small indistinctly nodular tumors were likely to contain both portal and arterial blood supplies, have portal tracts within their substance, and be comprised of uniform, well-differentiated cells. These authors considered the indistinctly nodular form to be the equivalent of carcinoma in situ, and they designated this as early HCC, noting the tendency to categorize such lesions as high-grade dysplasia in Western countries. In contrast, they considered distinctly nodular small HCC to represent an advanced cancer despite its small size.

Kanai et al. *(55, 56)* have additionally subdivided nodular HCC into three subtypes: type 1 is represented by HCC presenting as a single nodule, type 2 is a single nodule with extranodular growth, and type 3 has a contiguous multinodular growth pattern.

Blood groups have been related with macroscopic tumor patterns, with the suggestion that blood group status other than O was an independent risk factor for multinodular pattern HCC in those patients with tumor, and the presence of blood group O was associated with the solitary growth pattern *(46)*.

Portal vein thrombosis occurs in a high proportion of advanced cases *(57)*, and the frequency is lower in small HCC *(58)*. However, it has been proposed that curative resection may be possible, even in the presence of portal vein invasion, if the primary tumor is small, i.e., early stage *(59)*.

Less frequently, HCC may involve the main hepatic veins, the inferior vena cava or right atrium and can even extend into the large bile ducts. The clinical consequences of those involvements include Budd–Chiari syndrome, biliary obstruction, and hemobilia *(60–63)*.

Pathologic staging is a primary determinant of prognosis, and the growth pattern does not add additional information. However, the manner of growth, such as diffuse, may make it less likely that the tumor will be detected at an earlier stage, and, by definition, growth patterns such as diffuse or massive are synonymous with advanced disease and associated poor prognosis *(48, 49)*.

5.3. Staging of Hepatocellular Carcinoma

The International Union against Cancer and the American Joint Committee on Cancer (AJCC/UICC) published the Tumor-Node-Metastasis (TNM) pathologic classification for HCC in 1987 and later modified this in 2002 *(64)*. Most of the revisions were related to categorization of the primary tumor, i.e., T stage. A T1 tumor includes solitary tumors of any size without vascular invasion, and a T2 tumor includes solitary tumors of any size with vascular invasion. Multiple tumors are staged as either T2, in which the size of the largest tumor does not exceed 5 cm, or T3, in which the largest tumor

does exceed 5 cm in diameter. Factors such as bilateral location of tumors, or tumor multifocality versus intrahepatic metastasis of a single tumor, are not taken into account when assessing multiple tumors. Any tumor that involves a major branch of the portal vein (including portal vein and right and left branches) or hepatic vein (including right, left, and middle hepatic vein) is staged as T3. Finally, tumors with direct invasion of adjacent organs (excluding gallbladder) or penetration through the visceral peritoneum are staged as T4. A breakdown of the AJCC TNM Staging and Stage Grouping is provided in Tables 1 and 2.

The TNM system requires direct pathologic inspection of tumor extent and as such has limited usefulness in some clinical settings. A number of clinical or clinicopathologic staging systems have been proposed as offering more precise prognostic subgrouping and applicability for HCC patients who undergo hepatic resection, transarterial chemoembolization (TACE), or

Table 1
American Joint Committee on Cancer Staging for Intrahepatic Tumors: Definitions of TNM

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Solitary tumor without vascular invasion
T2	Solitary tumor with vascular invasion *or* multiple tumors none more than 5 cm
T3	Multiple tumors more than 5 cm *or* tumor involving a major branch of the portal or hepatic vein(s)
T4	Tumor(s) with direct invasion of adjacent organs other than the gallbladder *or* with perforation of visceral peritoneum
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastases
N1	Regional lymph node metastases
Distant metastases (M)	
MX	Distant metastases cannot be assessed
M0	No distant metastases
M1	Distant metastases

Table 2
American Joint Committee on Cancer Staging for Intrahepatic Tumors: Stage Grouping

Stage	*T*	*N*	*M*
I	1	0	0
II	2	0	0
IIIA	3	0	0
IIIB	4	0	0
IIIC	Any	1	0
IV	Any	Any	1

transplantation. Okuda et al. *(65)* developed a three-stage system with prognostic utility and based on tumor size, serum albumin level, presence of ascites and jaundice. The Cancer of the Liver Italian Program (CLIP) system uses the Child–Pugh score, tumor morphology, alpha-fetoprotein level, and portal vein thrombosis as independent predictive survival factors *(66)*. The Barcelona Clinic Liver Cancer (BCLC) Staging System is based on the presence or absence of symptoms, tumor multinodularity, vascular invasion, and extrahepatic spread *(67)*. The Chinese University Prognostic Index (CUPI) is constructed by adding liver function variables (total bilirubin, ascites, alkaline phosphatase, alpha-fetoprotein, and asymptomatic disease on presentation) into the TNM staging system *(68)*. The Prognostic Risk Score is based on vascular invasion (microscopic and macroscopic), lobar distribution, lymph node status, and largest tumor size *(69)*. The Japan Integrated Staging Score *(70)* incorporates a score for Child–Pugh category together with a score for TNM Stage as defined by the Liver Cancer Study Group of Japan. In this approach, the T stage is based on the variables of single versus multiple tumors, tumor size <2 cm, and absence of vascular invasion. HCC fulfilling all three of these criteria are T1, those fulfilling two factors are T2, those fulfilling one factor are T3, and those fulfilling no factors (i.e., multiple tumors, greater than 2 cm with vascular invasion) are considered T4. Final stage also incorporates node and metastasis status. Kudo et al. *(70)* found patient stratification by this approach to be superior to that obtained by the CLIP system.

Other variants, incorporating the Model for End-Stage Liver Disease (MELD) *(71)* criteria into baseline JIS *(72)* or CLIP *(73)* scoring systems, have also been described.

Several reports have compared the efficacies of multiple staging systems in a clinical setting. Cillo et al. *(74)* and Marrero et al. *(75)* found the BCLC staging system to be the best overall approach. In the setting of HCC treated with TACE, Georgiades et al. *(76)* found the nominal Child–Pugh results to

be the most accurate prognostic indicator. In contrast, Cho et al. *(77)* found the CLIP system to excel in this specific patient cohort. Seo et al. *(78)* found the CLIP system to have the best predictive power in a retrospective study.

In the United States at present (mid-2008), liver transplant candidates with either single intrahepatic HCC between 2 and 5 cm or two to three intrahepatic HCC each 3 cm or less in greatest dimension have been granted additional priority (22 points) within the MELD framework for liver transplantation. This approach, based on the Milan criteria proposed by Mazzaferro et al. *(79)*, has been criticized as being too restrictive *(80)*. Conversely, a retrospective study *(81)* of liver transplantation in the United States comparing the 5-year periods before versus after the introduction of the MELD priority exceptions for HCC showed a significantly worse survival for patients with HCC in the 3–5 cm range. Complementary approaches, such as those incorporating loss of heterozygosity analysis, may aid in delineating subgroups of HCC patients most likely to benefit from liver transplantation *(69, 82, 83)*.

5.4. Microscopic Pathology

Hepatocellular carcinomas can contain varied microscopic appearances, most of which recapitulate aspects of normal hepatocyte cytology and architecture. Well-differentiated HCC may be difficult or histologically impossible to distinguish from hepatocellular adenoma *(84–86)* and it may likewise be difficult to precisely establish the interface between tumor and normal liver. In contrast, poorly differentiated examples of HCC may betray only minor evidence of their hepatocellular origin.

The commonest architectural pattern of malignant hepatocytes is an arrangement that caricatures the normal trabecular arrangement of liver lobules (Fig. 6). These neoplastic pseudotrabeculae vary from 2 to over 20 cells in thickness, are irregularly arrayed, generally but not always have a reduced or absent reticulin framework, and are separated by a vascular network lined by endothelial cells and containing isolated arterial/arteriolar branches. In contrast, normal trabeculae are 1–2 cells thick, evenly arranged, bordered by a well-developed reticulin network, and separated by sinusoids without prominent endothelial cells.

Other growth patterns of HCC are variations on this basic theme. A pseudoglandular (pseudoacinar) pattern may result either from dilatation of the bile canaliculi between tumor cells or from central lytic degeneration of solid trabeculae. The gland-like spaces can be empty or contain PAS-positive cellular debris, lipid-laden macrophages, or bile. Complex pseudoglandular formations can result in pseudopapillary structures and give the appearance of "islands" of tumor cells, usually surrounded by a lining of endothelial cells *(87)*. A compact or solid pattern results when malignant cells appose each other closely, rendering sinusoidal or vascular spaces inapparent. It has

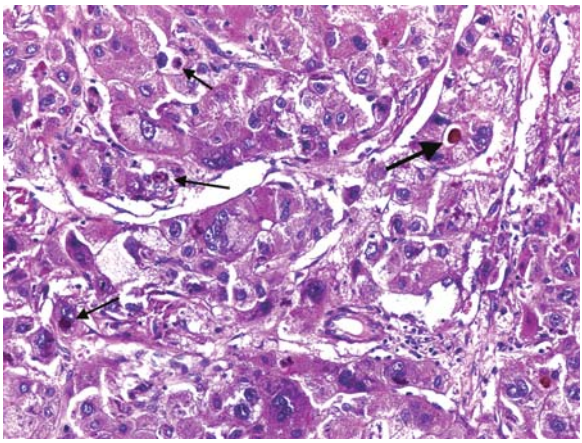

Fig. 6. Hepatocellular carcinoma. The tumor cells grow in distorted cords or trabeculae. Bile production (*large arrow*) and intracytoplasmic Mallory bodies (*small arrows*) are microscopic evidence of hepatocellular differentiation. More commonly, additional techniques are used to establish hepatocyte phenotype.

been suggested that hepatocellular carcinomas with a compact growth pattern have a better prognosis as compared with trabecular and acinar patterns *(88)*.

Tumor cells of HCC generally have more irregular nuclear membranes, coarser and more irregularly distributed heterochromatin, and a slightly higher nuclear:cytoplasmic ratios than do their benign counterparts. Mitotic and apoptotic activity are increased in the tumor cell population. As HCC approaches moderately to poorly differentiated phenotypes, there is a corresponding exaggeration of all of these features, with an increase in cell-to-cell heterogeneity and the emergence of giant and bizarre tumor cells in some cases. Different degrees of differentiation can be seen within a single tumor.

A variety of cytologic modifications may be seen within a given case of HCC. In general these have no prognostic relevance, but they can be useful clues for the diagnostic histopathologist. In some cases clear cells may predominate due to glycogen or lipid accumulation. Macrovesicular steatosis may be diffuse or focal and appears to be a more frequent finding in small HCC.

Bile pigment is noted in about 20% of hepatocellular carcinomas (Fig. 6). Bile within the neoplastic cells or bile canaliculi is an important indicator of hepatocellular origin. Bile is usually evident on routine histology, but on occasion it may be necessary to demonstrate bile canaliculi by polyclonal anti-carcinoembryonic antigen antibody which is cross-reactive with biliary glycoproteins (Fig. 7).

A variety of intracellular inclusions can be identified. Dense eosinophilic globular bodies may be intra- or extracellular. These are usually

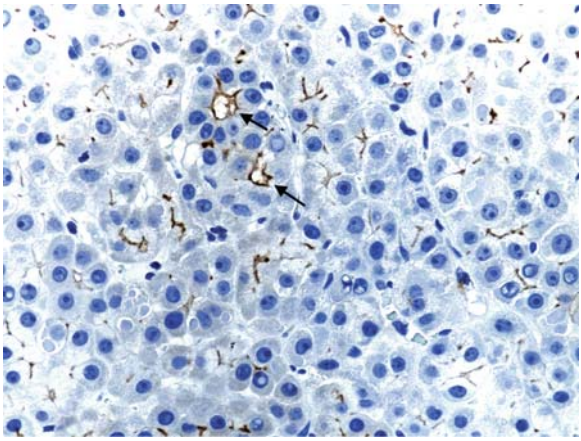

Fig. 7. Polyclonal carcinoembryonic immunostain highlighting bile canaliculi in hepatocellular carcinoma. In this well-differentiated tumor, the dark branch-like structures represent uptake of polyclonal CEA antibody, which cross-reacts with biliary glycoprotein. In some cases canalicular dilatation forms pseudoglandular structures (*arrows*). (polyclonal CEA immunostain with diaminobenzidine, 400×).

PAS-positive and can contain various proteins including alpha-fetoprotein, alpha-1-antitrypsin, alpha-1-antichymotrypsin, albumin, fibrinogen, and/or ferritin. Pale bodies are lightly staining, eosinophilic, intracytoplasmic inclusions that correspond to dilated rough endoplasmic reticulum and contain mainly fibrinogen, probably reflecting defective protein transport *(89)*. Pale bodies may simulate "ground glass" inclusions that are related to hepatitis B virus infection, but unlike true ground glass inclusions, they do not contain viral components *(90, 91)*. It has been suggested that proteins expressed in intracytoplasmic bodies might in some cases contribute to the malignant phenotype, since in one case p62, a phosphotyrosine-independent ligand of p56(lck) and putative signal transducer, was identified as the major component of such inclusions *(92)*. Typical Mallory bodies can be seen in about 20% of hepatocellular carcinomas, regardless of underlying disease *(93)*. Megamitochondria, enlarged lysosomes, myelin deposits, abnormal accumulations of glycogen, and degenerative material are occasionally seen and can be identified ultrastructurally. Copper, copper-related protein, and Dubin–Johnson-like pigment have all been described in tumor cells. The latter may impart a black macroscopic appearance to the tumor *(94)*. Rarely extramedullary hematopoiesis and granulomas can be detected.

Kupffer cells are present but quantitatively reduced in hepatocellular carcinomas, with more prominent decreases noted in larger and less well-differentiated tumors *(95)*. However, small, well-differentiated HCC may contain Kupffer cells in nearly normal numbers. Reduced Kupffer cell

function and cytokine production have been suggested as possible augmenters of HCC progression in an experimental animal model *(96)*.

The stroma of HCC is usually scanty. In some cases there can be a fibrous background and differentiation from other forms of adenocarcinoma may become problematic.

Tumor nodules are frequently surrounded by distinct fibrous capsules, and septum formation can be observed during the development of HCC. The capsule consists primarily of Type III collagen with Type I collagen facing the tumor in well-developed examples *(97–99)*. Small HCCs have a higher proportion of well-encapsulated tumors. The capsule and septa are mainly formed by alpha-smooth muscle actin-positive mesenchymal cells and can result from interactions between tumor and host liver parenchyma. It is thought by some that the capsule is a manifestation of host defense that can interfere with the growth and invasiveness of HCC *(97, 99)*. It has been suggested that tumor infiltration of the peritumoral capsule or of the surrounding parenchyma might correlate with a higher frequency of portal vein invasion and intrahepatic metastases *(48)*.

A four-tiered histologic grading system was originally devised by Edmondson and Steiner *(100)*, with Grades I–IV denoting progressive loss of differentiation. Tumor grades have been shown to correlate with the gross morphology, DNA content, proliferation markers, metastases, and AFP production but grading is a weak independent prognostic predictor *(101–103)*.

In our practice, about 15–20% of HCC behave in an aggressive fashion, despite small size. It is therefore incumbent upon the pathologist to assess each tumor for degree of differentiation and search for vascular invasion, regardless of tumor size. Whether such lesions have specific and early genetic or epigenetic changes that define such behavior remains to be determined.

5.5. Immunocytochemical Markers of Hepatocellular Carcinoma

A wide variety of antigens are detectable within HCC cells, and one recent textbook lists 109 such markers *(104)*. Some of these are of use in dissecting the various pathways of neoplastic progression that may occur in these tumors. Only a subset of markers has routine diagnostic applications and those are briefly considered herein.

Detection of alpha-fetoprotein expression is a classical approach to the diagnosis of HCC. The specificity of AFP is as high as 97%, but its sensitivity is low. Expression is often patchy and weak, and it has been suggested that AFP positivity correlates with size and differentiation of the tumor; small, well-differentiated HCCs are less positive than poorly differentiated ones.

This association apparently also extends to a lectin-reactive fraction of AFP (AFP-L3) that is currently used as a serum marker of HCC. Several studies have shown that serum AFP-L3-positive HCC patients have less well-differentiated tumors than do patients negative for this marker *(105, 106)*.

A number of other antibodies have long been used in the routine diagnostic evaluation of hepatocellular phenotype. Detection of biliary glycoprotein by the use of cross-reactive polyclonal anti-carcinoembryonic antigen (CEA) antibody highlights a bile canalicular pattern in 60–90% of HCC and was estimated in one series to be 79% sensitive and 97% specific for these tumors *(110)*. Adenocarcinomas and cholangiocarcinomas can show cytoplasmic staining with these antibodies, a pattern that is less common in HCC. Further, these other tumors can also react with the more specific monoclonal anti-CEA antibodies, a result that is only rarely seen with HCC when appropriate clones are used.

A canalicular pattern of staining in benign and malignant hepatocytes can also be demonstrated with antibody to CD10 (neprilysin) *(137, 138)*. In one study this antibody showed 68% sensitivity and 100% specificity for the differential diagnosis of HCC, although it did not distinguish it from normal liver parenchyma *(137)*.

HepPar 1 is a monoclonal antibody that detects the urea cycle enzyme carbamoyl phosphate synthetase 1 *(107)*. It decorates both benign and neoplastic liver cells and is not absolutely specific for the hepatocyte phenotype, as it may rarely be expressed in other cell and tumor types *(108, 109)*. However, in one study HepPar1 had 82% sensitivity and 90% specificity for the detection of hepatocellular carcinomas *(110)*. When it is used as a part of a diagnostic panel its diagnostic accuracy is enhanced *(110–113)*. HepPar-1 is more likely to be expressed in well-differentiated as opposed to poorly differentiated tumors.

For the differentiation of hepatocellular carcinoma from cholangiocarcinoma and metastatic carcinomas, particularly those of colorectal origin, immunostaining for individual cytokeratins is reportedly helpful. Normal adult liver cells contain cytokeratins 8 and 18 as defined in Moll's catalogue, and bile duct epithelial cells contain cytokeratins 7 and 19. At least in our experience this approach is less helpful than the use of other markers, since (a) hepatocytes can express CK7 when there is nearby fibrosis and this is particularly relevant with the scirrhous variant of HCC; (b) some HCC also express CK19, which is interpreted as showing a bipotential phenotype, although the tumor is still recognized as HCC; and (c) we have experienced significant artifactual staining with antibody to CK8. Of perhaps more utility is the use of cytokeratin antibodies to differentiate tumors of hepatocellular origin from colorectal adenocarcinoma. The latter are most often cytokeratin 20^{+} 7^{-}, a pattern rarely seen in either HCC or cholangiocarcinoma *(139)*.

Glypicans are a family of six heparin sulfate proteoglycans that are mainly expressed in a stage- and tissue-specific manner during development *(114)*. One form, glypican-3, is highly transcribed in hepatocellular carcinoma *(115)* and can serve as a marker for this tumor. Its use as part of a panel in the differentiation of HCC from hepatocellular dysplasia was considered above. It is not specific for HCC, with expression seen in about half of the cases of squamous cell lung carcinomas, liposarcomas, and nonseminomatous germ cell tumors *(116)* and in approximately 80% of melanomas *(117)*. In contrast to HepPar-1, glypican 3 is more sensitive in the detection of poorly differentiated as opposed to well-differentiated HCC *(116)*. Care in the use of this diagnostic marker is indicated, as it has been reported to be positive in 16% of preneoplastic nodular liver lesions *(116)* and also in 25 of 30 cases of benign liver tissue with prominent inflammation related to hepatitis C virus infection *(118)*.

β-Catenin translocation to the nucleus as a result of mutations or other aberrations of the β-catenin pathway is detectable in a minority of HCC, as is the expression of target gene products such as glutamine synthetase *(119)*. However, since these markers can also be expressed in a subset of hepatocellular adenomas, the diagnostic utility of these antibodies is somewhat limited. The possible prognostic significance of these markers remains unsettled at present.

Epithelial glycoprotein-2 is a cell surface molecule present on many carcinomas but absent on HCC *(140)*. The glycoprotein is detected by the monoclonal antibody MOC-31 and a positive staining result with this antibody would suggest a tumor other than HCC *(94)*.

Serum des-carboxy-prothrombin, also known as protein induced by vitamin K absence II (PIVKA-II) is useful as a marker of HCC. Immunohistochemical detection of this protein within the cytoplasm of HCC tumor cells was documented *(120)* and the authors suggested that it may prove useful in separating small HCC from examples of adenomatous hyperplasia. A separate study found an association with immunohistochemical detection of PIVKA-II within HCC and the presence of vascular invasion or higher tumor stage, suggesting its utility as a prognostic as well as a diagnostic marker *(121)*.

Gotoh et al. showed overexpression of osteopontin in HCC by quantitative PCR and immunohistochemistry *(122)*. This secreted glycoprotein is an organic component of the bone matrix, but is secreted by a number of other cell types. Osteopontin expression in HCC was associated with infiltration into the tumor capsule *(122)*, early tumor recurrence, metastasis, and lower survival *(123)*. Elevated serum levels of osteopontin had similar significance and were considered superior in one study to measurement of AFP or PIVKA-II *(124)*. Zhang et al. *(125)* showed that preoperative plasma osteopontin levels was an independent prognostic indicator of both overall and disease-free survival in a multivariate model.

Other potential prognostic immunohistochemical markers, such as galectin-3 *(126)*, survivin *(127)*, the stem cell markers CD133 *(128)* or EpCAM *(129)*, Aurora kinase B *(130)*, WT-1 *(131)*, histone deacetylase 1 *(132)*, phospho-ERK1/2 *(133)*, the transcription factor Twist *(134)*, mortalin (heat shock 70-kDa protein 9) *(135)*, the polycomb group oncogene Bmi-1 *(136)*, among others, are under active investigation at present.

Morphometric image analysis has been used to aid in the differential diagnosis of benign versus malignant hepatocellular lesions *(141–143)*. In one case, correlation of nuclear features with a specific loss of heterozygosity on 17p13 was reported *(144)*. Clinical application of these techniques, although promising, remains limited and the introduction of a more user-friendly technical infrastructure in the near future seems likely.

5.6. Molecular Pathology

The underlying molecular biology of HCC is covered elsewhere in this book and is not considered here. Likewise, specific cellular pathways of diagnostic pathologic importance for dysplastic lesions and hepatocellular adenomas are discussed above. Here we are concerned with the application of ancillary studies that may shed light on the behavior or prognosis of HCC beyond that discernible by the diagnostic histopathologist (in addition to potential prognostic markers already mentioned). Despite the impressive number of studies and the resultant large strides in understanding over the past decade, such approaches must still be considered to be early in evolution. These studies will eventually generate a comprehensive picture of HCC at the cellular and subcellular level which will in some cases confirm, and in other cases likely overthrow, our current concepts of this disease.

In the simplest hypothetical construct, cancer can be considered to represent an imbalance between cellular growth and cellular death. Thus, inappropriate activation of cell proliferation pathways and inhibition of apoptotic pathways could each tip the balance in favor of the tumor. Early studies of cellular proliferative markers, including S-phase fraction *(102)*, quantitation of silver staining nucleolar organizing regions (AgNORs) *(145)*, and immunohistochemical assessment of cell cycle proliferation antigens Ki67/MIB-1 or PCNA/cyclin *(145, 146)* all showed an inverse association with patient survival. Similar correlations extend to individual components of the cell cycle machinery. Overexpression of cyclin A and cyclin D1 was inversely associated with disease-free survival in some *(147, 148)* but not all *(149)* studies.

The application of microarray studies has upheld and expanded these studies. Lee et al. *(150)* examined cDNA derived from 91 HCC by unsupervised hierarchical clustering supplemented by additional analytic

procedures. They found two major subclasses of tumors that were strongly associated with patient survival, and increased translation of genes associated with cell proliferation was the strongest predictor of decreased survival.

Inhibition of apoptosis might be expected to stabilize a tumor population and serve as a negative prognostic indicator. In this regard Garcia et al. *(151)* used multivariate analysis to determine that a high level of immunohistochemical staining for the pro-apoptotic Bax protein was associated with a 31.9-month median survival whereas patients with weak or absent staining had 6.6-month median survival. Conversely, those patients with strong intratumoral expression of the antiapoptotic bcl-x had only a 5.8-month median survival, which increased to 32.7 months with strong expression. Nuclear expression of the antiapoptotic protein survivin was also associated with nuclear grade, microvascular invasion, proliferative rate, and local tumor recurrence as well as decreased survival in one study *(152)*. Lee et al. *(150)* also found a number of antiapoptotic molecules to be overexpressed in their poor survival group using a microarray approach. Similarly, telomerase activation serves to short-circuit normal cell senescence and subsequent cell death, and this protein is frequently activated in HCC *(153)*. High levels of telomerase activity are associated with recurrence following hepatectomy as well as decreased survival *(154, 155)*.

Disruption of cell cycle checkpoint proteins may facilitate genomic instability and the generation of tumor subclones with enhanced malignant behavior. The p53 tumor suppressor gene has been extensively studied in this regard *(156–165)* (reviewed in *(166)*). Immunohistochemical detection of p53 should be combined with p21 immunostaining to differentiate functional (p21 positive) from mutant (p21 negative) p53 expression. Additionally, some p53 mutations result in protein dysfunction without extended half-life and would therefore result in false-negative results by immunodetection. For these reasons, DNA mutation analysis is preferred. Mutations of p53 have generally been associated with disease recurrence and decreased survival *(166)*. P53 overexpression has also been associated with nuclear β-catenin expression and downregulation of E-cadherin in some studies *(167)* but not others *(168)*. Protein p73, which is an analogue of p53, also can induce apoptosis and in one immunohistochemical study was detectable in 32% of 193 HCC and found to be a correlate of poor prognosis *(103)*.

Aberrant retinoblastoma gene protein expression, including both absence and overexpression, was associated with poorly differentiated tumors and metastases in one study *(159)* and was felt to be a marker of advanced disease. Similar results were reported by this group for loss of the INK4 cyclin-dependent kinase (CDK) inhibitor p16 *(160)*. Inactivation of the INK4 CDK inhibitor p15 detected by promoter methylation-specific PCR was found in 64% of tumors in one study *(169)* and was associated with recurrence or

metastatic disease. This assay was also used to detect circulating tumor cells and the authors concluded that it might prove useful for both diagnostic and monitoring purposes.

Genomic instability may also manifest as increased aneuploidy and this has been associated with the degree of histologic differentiation *(101)* and decreased survival *(170)*. Markers of microsatellite instability have also been examined and in some studies have been associated with reduced disease-free survival *(171)*.

Composite markers of genetic alterations have been applied in a clinical setting. Marsh et al. *(83)* analyzed loss of heterozygosity at multiple loci to generate a fractional allelic loss index. Although this could not be used as a stand-alone assay due to the variability in the number of informative markers for a given tumor, these investigators were able to incorporate this information into a previously developed neural network model to accurately predict tumor recurrence in 81 of 81 evaluable patients. This approach, as well as comparative genomic hybridization *(172)*, has also found utility in distinguishing multiple independent primary HCC from intrahepatic metastases in some cases.

Microarray studies have generated a plethora of HCC-related data that must be integrated and simplified for clinical use. As examples, molecular signatures associated with intrahepatic versus extrahepatic metastasis *(173)*, vascular invasion *(174)*, clinical outcome including delineation of possible progenitor cell tumors *(175, 176)*, and recurrence following transplantation *(177)* represent some early results along these lines. Iizuka et al. *(178)* have recently presented a high-level overview of HCC-related microarray studies with a focus on current problems and challenges. The availability of high-throughput analysis of single nucleotide polymorphisms (SNP) will add an additional dimension to our ability to define HCC behavior. For example, SNP associated with high levels of alpha-fetoprotein production *(179)*, an adverse prognostic indicator, may eventually form part of a panel allowing a detailed clinicopathologic assessment of HCC. Such an approach will need to take into account the underlying etiologic factors, i.e., hepatitis B or C virus, aflatoxin, alcohol, as well as the presence or absence of cirrhosis, at a minimum.

6. PATHOLOGIC VARIANTS OF HEPATOCELLULAR CARCINOMA

6.1. Fibrolamellar Carcinoma

Fibrolamellar hepatocellular carcinoma (FL-HCC), also known as oncocytic hepatocellular carcinoma or polygonal cell-type hepatocellular carcinoma with fibrous stroma, is separable from ordinary hepatocellular

carcinoma on the basis of macroscopic, histologic, ultrastructural, and molecular features *(180)*. This distinctive variant of HCC is seen predominantly in young patients (90% under 35 years of age) without cirrhosis *(181)*. In a recent study using data from the Surveillance, Epidemiology, and End Results (SEER) program, El-Serag et al. *(182)* found this variant to comprise 13.4% of all primary liver cancers in patients under 40 years of age and 0.85% above this age. There appears to be a predominance in whites *(182)*, with relative rarity in Asia *(183)*, although it may be becoming more commonly recognized in that geographic area *(184)*. No sex predilection is known.

The clinical presentation is typically vague, with components of abdominal pain, malaise, and weight loss *(180)*. Less common presentations include biliary obstruction *(180)*, thrombophlebitis *(185)*, or massive bilateral metastatic spread to the ovaries (Krukenberg tumor) *(186)*.

The tumors are solitary in 90% of cases, ranging on average from 9 to 14 cm at time of presentation *(180)*. This neoplasm is unique among hepatocellular tumors in that the majority arise in the smaller left hepatic lobe *(104)*. The fibrous component of FL-HCC often forms a central scar that can be demonstrated by radiological techniques *(187, 188)*. The fibrous component also provides increased firmness to the tumor in comparison to typical HCC and may also be the site of calcification. The pattern of fibrous scar formation may superficially mimic that seen in focal nodular hyperplasia. It had been previously suggested that fibrolamellar HCC and focal nodular hyperplasia may be pathogenetically related, but most investigators do not subscribe to that concept *(189)*.

Microscopically, there is usually a compact architectural growth pattern but trabecular or acinar patterns can also be observed. The neoplastic cells are larger than normal hepatocytes (Fig. 8), polygonal in shape, and possess granular, eosinophilic cytoplasm, a so-called "oncocytic" appearance, due in fact to numerous swollen mitochondria *(190)*. Nuclei are vesicular, rounded, and have prominent nucleoli, the latter being a characteristic feature of this tumor. Mitoses are usually sparse; pleomorphism and multinucleation are infrequent. Tumor cells contain pale bodies that are reactive for fibrinogen and hyaline globular inclusion bodies may be present *(191)*. Intracellular bile production, fat, glycogen, copper and copper-associated protein can be detected *(192)*. In some tumors mucin production can be detected. Pseudoacinus formation may be seen, but the typical small glandular pattern associated with cholangiocarcinoma is not part of the normal spectrum of fibrolamellar HCC. Nevertheless, rare cases exist of fibrolamellar HCC combined with cholangiocarcinoma *(193)* or more typical HCC *(194)*. Clear cell changes have been described in a case of otherwise typical fibrolamellar HCC *(195)*.

Tumor cells are positive for HepPar-1 *(196, 197)* and hepatocyte cytokeratins 8 and 18 and may also contain biliary cytokeratins 7 and 19 *(180,*

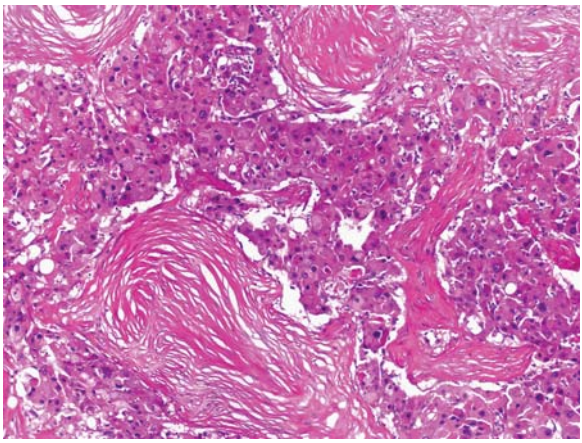

Fig. 8. Fibrolamellar hepatocellular carcinoma. In this variant, malignant cells contain plentiful cytoplasm and the tumor characteristically contains lamellated or layered areas of fibrosis (100×).

198). The tumor cells are usually reactive with antibodies to polyclonal CEA, alpha-1-antitrypsin, ferritin, and C-reactive protein. Alpha-fetoprotein is present in only occasional cases *(190, 199)*, and prominent AFP positivity, particularly when combined with elevated serum levels, suggests that a search for areas of more typical HCC should be undertaken *(200)*. Glypican-3 immunopositivity was seen in 64% of fibrolamellar HCC in one small series *(36)*, and in some cases uptake was patchy.

A prominent collagenous fibrous stroma that is arranged in thin parallel bands (lamellae) is a characteristic feature of fibrolamellar HCC (Fig. 8), but may be sparse or even absent in some tumors. The collagen is predominantly composed of types I, III, and V *(201)*. It has been suggested that lamellar fibrosis might be due to the production of collagen by stromal cells which in turn are stimulated by transforming growth factor-β (TGF-β) produced by tumor cells *(202)*.

Wilkens et al. *(203)* applied comparative genomic hybridization (CGH) to a series of HCC and found 1q amplification in one of two fibrolamellar HCC, with no changes in the other tumor. A separate study *(204)* also using CGH suggested that 4q+, 9p–, 16p–, and Xq– were more typical of fibrolamellar HCC than of other types of hepatocellular tumors. Fibrolamellar HCC is also marked by an absence of molecular alterations commonly found in other forms of HCC. These include an absence of TP53 mutations *(205)*, absence of β-catenin gene mutations *(206)*, and lack of survivin overexpression in fibrolamellar HCC in separate studies *(207)*. Fibrolamellar HCCs also show less promoter methylation than do HCC arising in cirrhotic livers *(208)*. However, 80–100% of fibrolamellar HCC in this study did show methylation of the CDH1 (e-cadherin) and RASSF1A (Ras association domain family 1

isoform A) genes *(208)*. The product of this latter gene is thought to act as a tumor suppressor by modulating a number of apoptotic and cell cycle checkpoint pathways *(209)*. Overexpression of the MAP kinase and phosphatidylinositol 3 kinase pathways in fibrolamellar HCC was detected in a separate DNA microarray study *(210)*. A number of other changes, including overexpression of the neurotensin gene, were also observed. This study again pointed to chromosome 1q as a significant locus for genetic alterations in this tumor.

Pure fibrolamellar HCC has a better prognosis than typical HCC primarily because it often presents as a surgically resectable lesion. For this reason, aggressive surgical management has been advocated for this tumor *(211–214)*. Resectability is an important prognostic variable *(215, 216)*, and Katzenstain et al. *(217)* concluded that resectability, not the fibrolamellar pattern, is the primary prognostic criterion, with patients presenting with an initially resectable lesion having a good prognosis regardless of histologic subtype.

6.2. Clear Cell Hepatocellular Carcinoma

Clear cell hepatocellular carcinoma is comprised of malignant hepatocytes, the large majority of which contain a clear or empty-appearing cytoplasm reflecting the accumulation of intracellular glycogen or lipid *(218, 219)*. The tumor typically arises in a background of cirrhotic liver, although it has rarely been reported in a noncirrhotic setting *(220)*. Liu et al. *(221)* found an association of clear cell change with hepatitis C virus infection in an Asian series, and individual associations with non-alcoholic steatohepatitis in a diabetic patient *(218)*, hypoglycemia, and hypercholesterolemia *(222)* have also been reported. One study *(223)* uncovered an example of clear cell HCC with a histologic appearance similar to that of chromophobe renal cell carcinoma. Since this tumor had significant microsatellite instability in contrast to the remainder of clear cell HCC in that series, the authors concluded that clear cell HCC represents a heterogeneous category of tumor. Orsatti et al. *(224)* also pointed to subtypes within this category. They showed that nondiploid clear cell tumors in their series were more pleomorphic and had a higher mitotic rate than diploid clear cell HCC and suggested that differences between these subgroups might account in part for differing opinions regarding the behavior of clear cell HCC.

One source of diagnostic difficulty lies in the possible histologic confusion with other tumors that may present as clear cell neoplasms, in particular renal cell carcinoma and adrenal cortical tumors. Immunohistochemical studies may be of aid in defining a hepatocellular phenotype of these lesions *(225)*.

Several series *(223, 226)* found no difference in overall clinical behavior between clear cell and typical HCC. In contrast, Liu et al. *(221)* reported

higher survival in clear cell versus common type HCC. They ascribed these differences to the more frequent presence of a tumor capsule and a lower rate of vascular invasion in the clear cell tumors. Jeon et al. *(227)* report the remarkable case of an elderly male who experienced spontaneous regression of a large clear cell HCC with metastases.

6.3. Scirrhous (Sclerosing) Hepatocellular Carcinoma

Scirrhous hepatocellular carcinoma is a rare variant of HCC that usually occurs in older age groups. It is reportedly associated with hypercalcemia in cases occurring in the United States but not in those reported from Japan *(228)*. Parathyroid hormone-related protein was detected by immunohistochemical means in tumor cells of one case and this was suggested as the cause of tumor-associated hypercalcemia *(229)*. The margin is often ill-defined on CT scan *(230)*. Macroscopically, the mass is usually large, firm, and gray-white in color. The characteristic histological features of the sclerosing hepatocellular carcinoma are nonlamellar, extensive fibrosis (Fig. 9) that extends from the sinusoidal areas *(231)* and a pseudoacinar formation of the tumor cells. Tumor capsule formation is seen in about 30% of cases *(230)* or less *(232)*, and in one series vascular involvement was more common than in typical HCC *(230)*. Origin within a dysplastic nodule has been described *(231)*.

The hepatocellular component of the tumor shows higher expression of cytokeratin 7 and lower expression of HepPar-1 than ordinary HCC

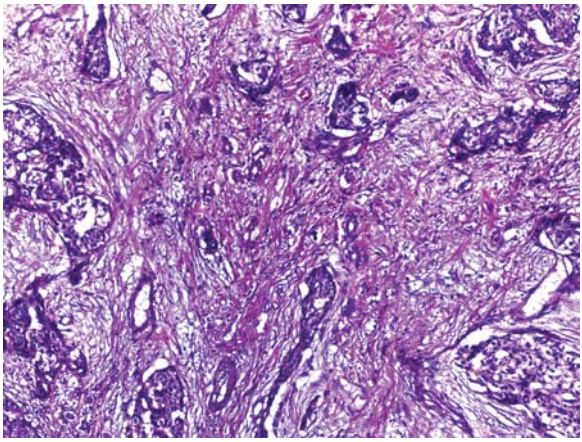

Fig. 9. Scirrhous hepatocellular carcinoma. In this variant, there is typically a diffuse fibrous background that simulates the pattern associated with cholangiocarcinoma. The malignant cells do not have the large appearance of the fibrolamellar variant, and ancillary techniques are usually required to identify them as having hepatocellular lineage (100×).

(233, 234). Frequent alpha-smooth muscle actin-positive-activated stellate cells have been described within this variant *(232)* and may contribute to the stromal changes. The sclerotic stroma, together with the occasional pseudoglandular pattern assumed by the tumor cells, may lead the diagnostic histopathologist to an incorrect diagnosis of cholangiocarcinoma. Okamura et al. *(235)* demonstrated that the stroma of scirrhous HCC lacks laminin-5 expression and shows only low levels of tenascin-C, both of which are highly expressed in cholangiocarcinoma. Further, stromal cells of scirrhous HCC are strongly alpha-smooth muscle actin positive, whereas those of cholangiocarcinoma reportedly have a more prominent glial fibrillary acidic protein-positive population *(235)*. Presence of intracellular mucin would also favor cholangiocarcinoma (or metastatic adenocarcinoma).

No significant clinical differences in the behavior of scirrhous HCC relative to ordinary HCC are known *(230)*.

6.4. Combined Hepatocellular/Cholangiocellular Carcinoma

Combined hepatocellular/cholangiocellular carcinoma is the least common type of primary epithelial liver cancer, accounting for approximately 2% of such tumors with reported frequencies ranging from 0.4 to 14.2% *(236)*. The World Health Organization defines this tumor as one that contains unequivocal elements of both hepatocellular carcinoma and cholangiocarcinoma that are intimately admixed, while also stipulating that this tumor be distinguished from synchronous intrahepatic hepatocellular carcinoma and cholangiocarcinoma that may also coexist adjacent to each other *(237, 238)*. Acceptable features of a hepatocellular component include the presence of bile, positivity for polyclonal carcinoembryonic antigen in a canalicular pattern, and/or demonstration of other hepatocyte marker such as alpha-fetoprotein *(239)* or HepPar-1. Demonstration of neutral epithelial mucin or cytokeratin 19 (and somewhat less specifically, cytokeratin 7) would suffice for demonstration of a biliary component.

Serum markers may mimic the mixed nature of the tumor, and concomitant elevations of AFP and CA19-9 may occur *(240)*. There are some purported differences in clinicopathologic features related to geographic area *(236)*. In Asian series, these tumors have been more often associated with underlying chronic liver disease and hepatitis B virus infection, whereas in Western series, more examples occur in the absence of chronic liver disease. This has practical implications, as patients without cirrhosis are more likely to qualify as resection candidates.

The tumors morphologically consist of mixed populations of hepatocytes, neoplastic cholangiolar cells, and small undifferentiated intermediate or oval-like cells on the basis of both light and electron microscopy *(241)*.

Characteristically, areas of trabecular hepatocellular carcinoma are mixed with varying numbers of bile duct-type cells (Fig. 10a). Generally the central areas are typical of hepatocellular carcinoma and the peripheral cells resemble biliary-type cells. In other cases there may be distinctive nodules of differing appearance, and in yet other examples the two histologic phenotypes may be finely mixed *(242)*. There is a variable component of stromal fibrosis and mixed neutrophilic and lymphocytic inflammation that is usually related to the cholangiolar component *(243)*. A proportion of combined hepatocellular/cholangiocellular carcinomas can be associated with a sarcomatoid component *(241, 244)* (Fig. 10b).

Opinions regarding the pathogenesis of combined HCC/CC ascribe it variously to metaplasia of pre-existent HCC into cholangiocarcinoma *(242)* or to a bipotential progenitor cell capable of giving rise to both components *(245)*.

The two components of HCC/CC do share a number of features. Imai et al. *(246)* showed similar p53 and RB-1 locus mutations in both hepatocellular and cholangiocellular components of mixed HCC/CC in some patients. A cell line derived from a human HCC/CC showed features of one or the other cell component dependent on growth conditions *(247)*. Gil-Benso et al. *(248)* were able to derive in vitro rat hepatocellular, cholangiocellular, and oval type cell lines from a single founder cell line derived from a rat HCC/CC. These lines showed similar molecular genetic alterations.

Immunophenotypic analysis of HCC/CC also discloses a subpopulation of cells corresponding to intermediate- or small-sized cells that contain

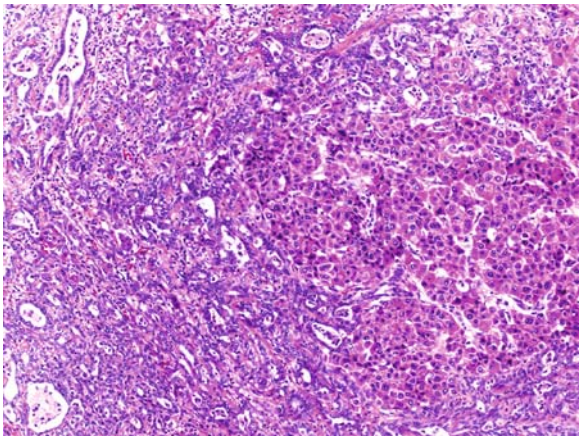

Fig. 10a. Mixed hepatocellular and cholangiocarcinoma. This tumor shows solid areas of cells resembling hepatocytes on the right side of the photograph, whereas smaller cells with significant gland formation largely populate the left side. The immunophenotype of these areas also varied between hepatocellular and biliary (100×).

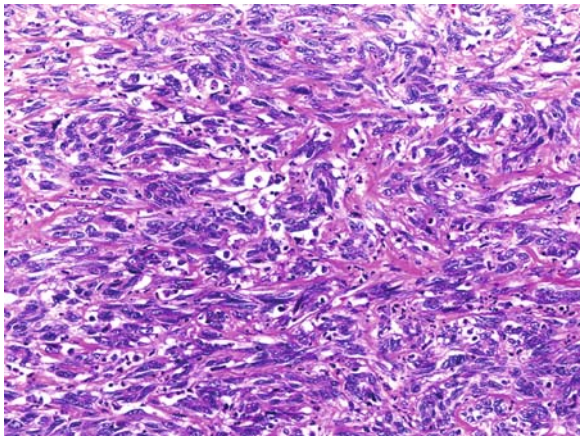

Fig. 10b. Sarcomatoid change in mixed hepatocellular/cholangiocarcinoma. This is a separate area of the tumor shown in Fig. 10a. In this region the neoplastic cells have a spindled or "streaming" appearance that is usually found in sarcomas. These cells expressed both vimentin and cytokeratins, supporting the concept that they arose by a form of metaplasia or tumor progression (or "dedifferentiation") from the epithelial elements in the tumor.

biphenotypic markers. Zhang et al. *(245)* found that these cells coexpress HepPar-1 and CK19 by double immunofluorescence studies and also found similar results using a combination of OV-6 and c-kit. (The presence of c-kit in 83% of their tumors also led them to suggest investigation of Gleevec therapy in these tumors.) They interpreted these cells as putative progenitor cells. These cells are not diagnostic for combined HCC/CC, as similar cells have been described in dysplastic foci.

Aishima et al. *(243)* examined a series of small (<3 cm) HCC/CC and found that those in which the biliary component coexpressed CK19 and mucin had worse survival and more frequent tumor recurrence than did those without these two markers.

Related studies raise the possibility that a limited form of biphenotypic expression may be more widespread than commonly appreciated. Dumez et al. found that 28% of 107 otherwise typical hepatocellular carcinomas expressed CK7 and/or CK19. Those expressing the biliary marker CK19, but not those expressing CK7, had a higher recurrence rate.

6.5. Sarcomatoid Hepatocellular Carcinoma

Sarcomatoid hepatocellular carcinoma is a rare variant of HCC that may contain spindle-shaped cells with features of any of a variety of sarcomas *(249, 250)*, including fibrosarcoma, leiomyosarcoma, rhabdomyosarcoma, osteosarcoma, and others. Oscteoclast-like or anaplastic giant cells may also

be seen, with the former cell type thought to represent benign reactive histiocytes *(251)*. A malignant hepatocellular component is present, and rarely this may take the form of hepatoblastoma *(252)*. The sarcomatoid component is considered to represent a form of tumor progression, or "dedifferentiation" of the epithelial component, as attested to by the demonstration of hepatocyte keratin subtypes or alpha-fetoprotein positivity reported in the sarcomatous elements in some cases *(249, 253)*. Haratake et al. *(253)* suggest the keratin 8 positivity in the sarcomatoid element may be diagnostically helpful in distinguishing these tumors from true intrahepatic sarcomas.

Park et al. *(254)* examined expression of the transcription factor SRF (serum response factor, c-fos serum element-binding transcription factor) in HCC. This protein regulates expression of a number of genes and is thought to play an important role in mesoderm development during embryogenesis *(255)*. SRF expression was found to be prominently expressed in high-grade HCC, especially sarcomatoid HCC. They proposed that this protein activated genes that contributed to acquisition of a mesenchymal phenotype, thereby contributing to tumor progression.

Sarcomatoid change can also occur in mixed hepatocellular–cholangiocellular tumors as noted above (Fig. 10b) *(241, 244)*, and the relationship between those tumors and sarcomatoid HCC is currently undefined.

Given the rarity of this variant, most conclusions regarding survival are based on single case reports or small series and appear to follow the course expected of a high-grade malignancy.

7. HEPATOBLASTOMA

7.1. Clinical Aspects

Hepatoblastoma is the most common primary liver tumor of infancy and childhood. It arises most frequently during the first 5 years of life and may rarely be diagnosed in the fetus *(256)*. Rare cases are reported in adults *(257–259)*. The male:female ratio for hepatoblastoma is approximately 3:1 and the tumor can be associated with several congenital abnormalities, including hemihypertrophy, Beckwith–Wiedemann syndrome, familial colonic polyposis, cardiac and renal malformations, Noonan syndrome *(260)*, and glycogen storage disease type IA *(261–265)*. There is no known relationship with liver cirrhosis.

Clinically, a rapidly enlarging upper quadrant mass, vomiting, and/or fever are frequent presenting signs and symptoms. Serum alpha-fetoprotein is elevated in approximately 90% of patients. In infants and children with a primary liver tumor, low levels of AFP suggest the presence of either well-differentiated or immature hepatoblastoma or fibrolamellar hepatocellular

carcinoma. In occasional cases, HCG production may occur and may be sufficient to cause virilization *(266)*.

7.2. Macroscopic Pathology

Macroscopically, the tumor usually presents as a single, well-circumscribed, large mass up to 25 cm. The gross tumor appearance may be heterogeneous due to any combination of necrosis, hemorrhage, calcification, and cystic degeneration. The presence of a mesenchymal component in some tumors may also contribute to this variability.

7.3. Microscopic Pathology and Ancillary Studies

There are several histologic patterns that segregate into pure epithelial type and mixed epithelial–mesenchymal type. The epithelial type is further categorized based on the appearance of the cells into fetal, embryonal, small cell undifferentiated, or macrotrabecular patterns. These patterns may occur alone or in combination.

Fetal type cells bear a resemblance to normal fetal liver cells with granular cytoplasm, round to oval centrally placed nuclei and single small nucleoli. Mitoses are scant. The cytoplasm may contain fat and glycogen. They may assemble in irregular cords that are two cells in thickness and contain bile canaliculi and sinusoids *(265)*. Embryonal type cells are small and elongated with hyperchromatic nuclei and scant cytoplasm. Mitoses can easily be detected and foci of necrosis can also be present. The cells are arranged in ribbons, cords, or rosettes *(267)*. Fetal- and embryonal-type hepatoblastomas in particular commonly show foci of extramedullary hematopoiesis *(268)*. The small cell undifferentiated variant is comprised of small, round, and loosely arranged cells that are histologically similar to those of other pediatric "small blue round cell tumors" *(199, 208, 209, 269–271)*. Enlarged, bizarre cells may also occur and mucoid stroma can be associated with the small cell variant *(272)*. The macrotrabecular pattern differs in that this term refers to architecture, not cell appearance, and consists of thick columns, or trabeculae, of fetal or embryonal cells or of cells resembling those of typical HCC.

Mixed-type hepatoblastomas combine the epithelial elements listed above with a metasplastic mesenchymal component that characteristically has a spindled, undifferentiated appearance. Osteoid is also frequently present. Other components such as cartilage, bone, striated muscle, neural tissue, respiratory or intestinal type epithelial cells, and other mature tissues may occur in some tumors and this combination of tissues gives rise to what has been termed teratoid hepatoblastoma *(273)*.

Hepatoblastomas typically express AFP in epithelial cells, especially in fetal and embryonal variants. Other markers of hepatocellular phenotype, such as HepPar-1 *(108, 110, 274)* and glypican-3 *(275)*, are also expressed. Hepatocyte cytokeratins 8 and 18 are expressed; in addition, cytokeratin 7 expression may occur in small epithelial cells in association with albumin expression, suggesting a stem-like or bipotential cell population *(276)*. Fiegel et al. *(277)* examined a series of hepatoblastomas for expression of stem cell and hepatic or biliary lineage markers and concluded that a stem-like population of cells existed within duct-like structures in the tumors. Phenotypic plasticity may play a role in the development of mesenchymal components of these tumors, and this is reflected in immunophenotype. For example, HCG positivity can be detected in giant cells *(278)*, and vimentin is positive in anaplastic cells and osteoid. It should also be noted that the mesenchymal elements generally retain cytokeratin expression, which belies their epithelial origin. From a practical diagnostic perspective, such variability may present difficulties when a diagnosis must be rendered on a small sample such as a needle biopsy. Ramsay et al. *(279)* observed that such samples could focally express antigens such as CD99, CD56, desmin, or PGP9.5 that are usually associated with other pediatric neoplasms such as small round cell tumors.

Similar to HCC, hepatoblastomas may show β-catenin activation. Curia et al. *(280)* showed mutations in this gene in 19% of sporadic hepatoblastomas in their series, but also demonstrated nuclear accumulation of this protein in 67%, suggesting separate alterations in this pathway in individual cases. This group also found p53 mutations in 24% of cases, and evidence of microsatellite instability in 81% of tumors in their series. They were unable to associate these findings with specific histologic subtypes. A discrepancy between the low frequency of detectable β-catenin gene mutations and the ubiquitous accumulation of this protein was also observed in the study of Yamaoka et al. *(281)*.

Intranuclear accumulation of β-catenin was also observed in both pre- and post-treatment biopsies of hepatoblastomas in another study *(282)*. In contrast, aberrant cytoplasmic localization of the hepatocyte growth factor receptor Met, present in pretreatment biopsies, showed a decreased uptake following treatment in 85% of cases. This led the authors to suggest that Met might have a significant role to play in the pathogenesis of this tumor *(282)*.

Hepatoblastomas with embryonal and/or small cell components show significantly higher expression of the FOXG1 (human forkhead box G1) protein than do purely fetal–epithelial-type tumors *(283)*. This protein, which is one component of a large family of transcription factors with diverse actions *(284)*, may be associated with repression of TGFβ-1-induced p21 expression, and these authors suggested that it may contribute to the undifferentiated state in hepatoblastomas.

Delta-like protein (DLK/Pref-1) is a membrane protein expressed in normal hepatoblasts *(285)* and it has found recent use as a marker to define and isolate these progenitor cells *(286)*. Deszo et al. found expression of this marker in 100% of 31 hepatoblastomas by immunohistochemical staining and recommended it as a potential marker for these tumors. In the global microarray gene expression study of Luo et al. *(115)*, DLK was one of several genes with prominent increased expression in a subset of hepatoblastomas relative to HCC. Other overexpressed genes included mitogen-inducible gene 6 (Mig6) and TGFβ-1. IGF2 was also overexpressed in a subset of hepatoblastomas relative to HCC. In vitro studies support the concept that this can act as a growth factor for hepatoblastoma via the IGF-I receptor and PI3 kinase, and this pathway may be a good target for molecular therapy *(287)*.

7.4. Staging and Prognosis

In contrast to staging of HCC, staging of hepatoblastoma incorporates the results of surgery. Postsurgical Stage I disease implies complete tumor resection, Stage II includes those patients with postsurgical microscopic residual disease, tumor spill, or rupture at surgery. Stage III patients have unresectable tumor or gross residual tumor or positive lymph nodes and Stage IV is defined by the presence of distant metastases. The U.S. National Cancer Institute estimates the present cure rate at over 90% for Stages I and II, 60% for Stage III, and approximately 20% for Stage IV. Austin et al. *(288)* recently reviewed the United Network for Organ Sharing (UNOS) database and found that liver transplantation for unresectable hepatoblastomas was associated with 66% actuarial 10-year survival, with 54% of deaths related to recurrent or metastatic disease.

In addition to stage, a low serum alpha-fetoprotein level is viewed as a poor prognostic indicator. In the series of D'Antiga et al. *(289)*, patients with multifocal hepatoblastoma in association with AFP <100 ng/ml survived only with transplantation. De Ioris et al. *(290)*. found 9 of 15 patients with serum AFP below this level and with evaluable histology had a small cell undifferentiated epithelial component, and the overall 2-year survival in their patients with low AFP level was 24%.

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7 Hepatocellular Carcinoma Associated with Hepatitis B Virus

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CONTENTS

ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms in the world. Current data indicate that hepatitis B virus (HBV) and hepatitis C virus (HCV) are the most significant hepatocarcinogens for the majority of HCCs in the world. Globally about 80% of HCC is considered to be causally associated with chronic infection with HBV. Although HCC is rare in the United States, it is perhaps the most prevalent cancer in Asia and West Africa where the prevalence of HBV infection is high. Without proper intervention for HBV infection, the HBV carriers are at risk for developing HCC. In a large prospective study of 3,653 HBV carriers in Taiwan, 164 persons developed HCC in a 12-year follow-up period; the most

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important risk factors for HCC were increased baseline and persistently elevated HBV DNA levels. During the last decade, great strides have been made in the treatment of HBV infection. Prospective and retrospective studies of large numbers of chronic hepatitis B patients with advanced liver disease have demonstrated that the treatment with anti-HBV agent not only delayed the disease progression but also reduced the incidence of HCC.

For HBV-related HCC, primary prevention of HCC is vaccination for all uninfected individuals. Secondary prevention of HCC should focus on those who are already infected. By careful monitoring and discreet antiviral therapy with available antiviral agents we should strive to prevent the development of HCC by suppression of viral replication, elimination of the virus, and thereby delay/prevent the development of HCC in HBV-infected patients.

HBxAg is a *trans*-activating protein that stimulates virus gene expression and replication, thereby promoting the development and persistence of the carrier state. HBxAg also alters patterns of host gene expression that contribute importantly to the pathogenesis of chronic liver diseases and the appearance of HCC. HBxAg blocks immune-mediated apoptosis by Fas and TNF alpha, thereby promoting the persistence of virus-infected cells. HBxAg promotes the development of fibrosis at multiple steps, thereby giving rise to intrahepatic lesions from which HCC appears. Finally, HBxAg activates host genes that support hepatocellular growth and survival and downregulates a number of tumor suppressor pathways that normally keep the growth of cells in check. Due to these and other varied activities, it is likely that HBxAg will be a very important target for the development of novel therapeutics against hepatitis B in the future.

Key Words: HCC; HBV; HBV-DNA; HCV; HBsAg; HBeAg; antiviral drugs; lamivudine; adefovir; entecavir; telbivudine; tenofovir; interferon; risk factors; HBxAg; trans-activator; Fas; TNF alpha; beta-catenin; fibrosis; DNA methylation; tumor suppressors

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms in the world responsible for 598,000 deaths annually *(1)*. Although HCC is rare in the United States, it is perhaps the most prevalent cancer in Asia and West Africa where the prevalence of chronic hepatitis B infection is high. The vast majority of deaths from HCC occur in East Asia and Africa. Current data indicate that hepatitis B virus (HBV) and hepatitis C virus (HCV) are the most significant hepatocarcinogens for the majority of HCCs in the world *(2–4)*. Globally about 80% of HCC is considered to be causally associated with chronic infection with HBV *(2, 5, 6)*, and, among the remaining non-HBV-associated HCCs, the majority are associated with chronic infection with HCV *(7, 8, 9)*. However, there are HCCs for which the causes are still unknown *(10, 11)*.

1. HBV AND HCC

HBV was first discovered in 1965 by Blumberg et al. *(12)*. He named the new antigen the "Australia antigen" since it was found in the blood of an Australian Aborigine during Blumberg's long pursuit for human polymorphism. Australia antigen was later found to be the envelope protein of the whole virus (called Dane particle) *(13)* and was renamed as Hepatitis B surface antigen (HBsAg). A large number of epidemiologic studies, including those of Blumberg's group *(14–17)* and of others *(18, 19)*, further documented the causal association of HCC with HBV. Later, the landmark study by Beasley et al. *(20)* lucidly demonstrated the relationship between HBV and HCC.

Half of the world's population live in the regions of high incidence for HCC. These regions also coincide with endemic regions for HBV infection with a carrier rate ranging from 11.4 to 24.5% in China, Taiwan, and Vietnam *(3)*. It has been known that nearly 350 million people in the world have chronic HBV infection. Of these carriers more than half live in Asia. Datamonitor report of 2002 *(21)* shows the approximate numbers of HBV carriers in the world (Table 1) and the prevalence rates of HBV infection around the world (Table 2).

Table 1
Chronic HBV Carriers in the World (2001)

	Countries	*In millions*		*Countries*	*In millions*
Americas	USA	1.2	**Asia**	China	123.7
	Brazil	3.7		India	30–50
	Dominican Republic	2.9		Indonesia	13.3
	Mexico	1.0		Philippines	8.1
Europe		>6.7		Thailand	5.0
	Turkey	2.7		Japan	3.7
	Italy	1.5		S. Korea	2.8
	Spain	0.6		Taiwan	2–2.6
	Germany	0.5		Malaysia	1.2
	Greece	0.3		Singapore	0.2
	France	0.3	**Africa**	Egypt	2.9
	United Kingdom	0.6			

Datamonitor Report DMHC1802, 2002 *(21)*

As shown in Tables 1 and 2, the prevalence of hepatitis B varies markedly around the world. In highly endemic regions, such as China, Vietnam, Taiwan, other Southeast Asian countries, and much of Africa, 8% or more of the population are chronic HBV carriers *(3, 22, 23)*. Regions of intermediate

Table 2
Prevalence of HBV Infection in Asian Countries

Countries	*%*
China	8–20
Hong Kong	10
India	4.7
Korea	5–6
Malaysia	5.2
Philippines	12–15
Taiwan	15–20
Thailand	8–10
Vietnam	10–20

Datamonitor Report 2002

prevalence include parts of Southern and Eastern Europe, the Middle East, Japan, the Indian subcontinent, much of the former Soviet Union, and Northern Africa. Regions of low prevalence include North America, Western Europe, certain parts of South America, and Australia *(21)*.

The skewed distribution of HBV infection is due to the different modes of transmission. In the hyper-endemic regions, perinatal transmission and horizontal spread among children are the major sources of infection. On the other hand, in the low-endemic regions, horizontal transmission through sexual activity among young adults and intravenous drug use are the major modes of transmission *(24)*.

Transmission of infection from an HBV carrier mother to neonate has accounted for the majority of infections in the endemic areas. Eighty-five percent of hepatitis B surface antigen (HBsAg)-positive mothers who are hepatitis B e antigen positive will transmit the disease to their offspring whereas mothers who are positive for antibody to HBeAg (anti-HBe) do so much less frequently (31%) *(25)*. More importantly, the chance of becoming a chronic carrier inversely correlates with the time of infection. If infection occurred at birth, nearly 90% will become a chronic carrier, during infancy 50%, during early childhood under 5 years of age 20%, and thereafter 5–10% will become chronic carriers of HBV *(2)*. Other less frequent sources of infection include household contact with an HBV carrier *(26)*, hemodialysis *(27)*, exposure to infected health-care workers *(28)*, tattooing, body piercing *(29)*, artificial insemination *(30)*, and receipt of blood products or organs *(31)*.

Without proper intervention for HBV infection, 15–40% of these HBV carriers are at risk for developing cirrhosis and/or HCC, which could lead to death *(32)*.

Evidence suggests that it takes 20–40 years to develop HCC from the time of infection with HBV *(20)*. Most HCC patients are between the ages of 40 and 60 years although there are some exceptions including childhood HCC in Taiwan *(33)*. Furthermore, young adults with HBV-related HCC, such as 20 and 27 years old, have been observed (Hann, personal communication). In the majority of patients with HBV-related HCCs, the time of infection can be traced back to the perinatal period and/or to early childhood *(3, 14, 17, 34)*.

2. HBV AND PATHOGENESIS OF HCC

Hepatitis B virus is a 42 nm (in diameter) double-stranded DNA virus belonging to the hepadna virus (hepatotropic DNA virus) family, which includes hepatotropic viruses that infect woodchucks, squirrels, and ducks *(35–37)*. The intact virus, referred to as the Dane particle, consists of an outer coat component of hepatitis B surface antigen and an inner core component of hepatitis B core antigen (HBcAg) *(12, 13, 38)*. During hepatitis B infection, a breakdown product of core, the hepatitis B e antigen (HBeAg), circulates in the blood which indicates an active viral replication in the liver cells and therefore, a relatively increased state of infectivity *(39, 40)*. The hepatitis B viral genome is approximately 3,200 base pairs in length, is partially double stranded, and uses a retroviral mode of replication *(41)*. The viral genome contains genes that code for HBsAg, HBcAg, and DNA polymerase. In addition, there are pre-S regions that code for proteins thought to be involved in the process of viral attachment to the hepatocytes during infection. An additional X gene codes for a protein whose function has not yet been fully defined *(42–44)* but is suspected to be linked with hepatocarcinogenesis *(45, 46)*.

The postulated pathogenesis of HCC by HBV will be further discussed in later part of this chapter by Feitelson. Most HCCs are associated with chronic liver disease, including hepatocyte necrosis and active inflammation (chronic active hepatitis) or fibroblastic proliferation (cirrhosis) *(2)*. HCC is considered to be a long-term, multistage disease process encompassing multiple genetic alterations, including activation of cellular oncogenes and inactivation of tumor suppressor genes *(47, 48)*.

3. INCIDENCE OF HCC

The rate of HCC development varies widely between the endemic regions and non-endemic regions. In China, for instance, the incidence is 52.1/100,000 while it is only 5.1/100,000 in Northern European countries. The incidence of HCC is significantly high in southeastern Asia, sub-Saharan Africa, and Melanesia. More than 80% of HCC cases are observed in these areas, in China alone accounting for 55% of HCC in the world *(1)*.

During the past decades, the incidence of HCC has decreased in some areas in East Asia such as in Shanghai, Singapore, and Hong Kong. It is believed that effective HBV vaccination programs may have contributed to the reduction. However, the opposite phenomenon was reported in some countries in Europe, North America, and Oceania. The increasing incidence of HCC in some western countries is attributed to the increase in HCV infection and also the immigration of people from endemic regions who are already infected in their home countries.

4. HCC IN ASIAN AMERICANS

Increasing incidence of HCC in the United States is clearly illustrated by the HCC incidence in California, the state which has the largest number of Asian Americans *(49)*. As shown in Table 3, in contrast to non-Asians, liver cancer is one of the five most common cancers for Asian American males. This high incidence of HCC among Asian Americans is attributed to the high prevalence rate of HBV infection among Asian Americans *(50)* as shown in Table 4.

Over the last three decades, a large number of Asians have migrated to the United States and have brought with them their high HBV carrier rate and the risk for chronic hepatitis B, cirrhosis, and HCC. In fact the HBV carrier rate among the first-generation immigrants is similar to that of people living in their native lands *(51–54)*.

Table 3
Five Most Common Cancers in Males by Race/Ethnicity California, 1997–2001

	Rank				
	1	*2*	*3*	*4*	*5*
Asians					
Laotians	**Liver**	Lung	Stomach	Colorectal	Oral
Cambodians	Lung	**Liver**	Colorectal	Lymphoma	Oral
Vietnamese	Lung	**Liver**	Prostate	Colorectal	Stomach
Chinese	Prostate	Colorectal	Lung	**Liver**	Stomach
Korean	Lung	Stomach	Colorectal	Prostate	**Liver**
Philippino	Prostate	Lung	Colorectal	Lymphoma	**Liver**
Non-Asians					
White	Prostate	Lung	Colorectal	Bladder	Melanoma
Hispanic	Prostate	Colorectal	Lung	Lymphoma	Leukemia
Black	Prostate	Lung	Colorectal	Lymphoma	Oral

California Cancer Facts and Figures 2005, American Cancer Society

Table 4
HBV Infection Among Asian Americans

	%
Cambodian	15
China	11
Hmong	16
Indonesia	11
Japan	2
Korea	7
Laos	12
Malaysia	11
Philippines	8
Taiwan	11
Thailand	14
Vietnam	14

Tong and Hwang *(50)*

In the United States there are 1.4–2 million HBV carriers *(55, 56)*. According to a US census Bureau report, there are about 12.9 million Asian and Pacific Islanders in the United States *(57)*. Although Asian Americans constitute only 4.5% of the total US population of 290 million, they constitute nearly half of the total US HBV chronic carriers, largely due to the influx of infected people from the endemic regions *(50, 55)*.

Currently, the estimated prevalence rate of chronic HBV carriers ranges between 5 and 15% among Asian Pacific Americans *(50)*. This estimate is further confirmed by a recent cross-sectional survey of 5,341 Asian Americans (10.5%) *(58)* and 6,130 Korean Americans (7%) *(54)*. In contrast, HBV carrier rate for the general US population is less than 0.3%.

For Asian Americans, chronic hepatitis, cirrhosis, and HCC constitute significant morbidity and mortality, most of which are attributed to chronic HBV infection. With the high prevalence rate of chronic HBV infection, without intervention, 15–40% will develop serious sequelae including HCC during their lifetimes. Fortunately, the lengthy interval between the infection and the development of HCC provides an advantage for clinicians to intervene and delay the progression of the disease.

Although the chronic HBV carriers have been infected for long, most of these patients do not have symptoms. It has been the common experience that many patients are found to have chronic hepatitis B incidentally during a routine screening. Among 139 incidentally identified HBsAg (+) Korean Americans, 11% were found to have cirrhosis and 42% to have active

hepatitis on complete evaluation including liver imaging studies and complete liver profiles *(54)*. Tong and Hwang conducted a prospective study of 207 HBsAg (+) Asian American patients with chronic hepatitis *(50)*. During an average follow-up period of 3.3 years, eight patients developed HCC; the calculated incidence of HCC in these Asian American patients with chronic hepatitis B was 3865/100,000. This is much higher than those reported in Taiwan by Beasley (495/100,000) *(2)* and by Liaw et al. (826/100,000 for all ages and 2768/100,000 for patients older than 35 years of age) *(59)*. Nonetheless, it is important to point out that Beasley followed asymptomatic carriers, and Tong et al. and Liaw et al. followed chronic hepatitis B patients.

5. PREVENTION OF HCC

5.1. Primary Prevention of HCC

Primary prevention would be universal vaccination for all uninfected individuals. The nationalized vaccination program in Taiwan clearly reduced the incidence of HCC in children as reported by Chang et al. *(22)*.

5.2. The Secondary Prevention of HCC

The secondary prevention of HCC will be to effectively interrupt the progression of HBV infection to cirrhosis and HCC by active antiviral therapy. No doubt that the ultimate goal of treatment for chronic hepatitis B is to prevent the development of HCC.

5.3. Risk Factors for the Development of HCC

It is important to understand that not all HBV carriers develop HCC. Table 5 illustrates the putative risk factors. Patients who have already developed cirrhosis are at high risk for developing HCC. Patients of Asian background are at high risk because they are likely to have been infected early in life. Males have a higher incidence rate for HCC among HBV carriers with the 4:1 ratio in male to female *(2)*. The biologic basis for the gender difference in the risk for HCC is not well elucidated; however, male hormones *(2)*, differences in body iron storage *(60)*, and differences in behavior, such as drinking and smoking *(61)*, have been considered to be contributory factors. Age greater than 40 years is also considered a risk factor, since HCC occurs most commonly later in life. It is estimated that, among male HBsAg (+) carriers older than 40 years, 40–50% will eventually succumb to cirrhosis or HCC *(2)*. As to the iron storage, a sustained serum ferritin level greater than 300 ng/ml is considered a risk factor. In a longitudinal follow-up study of 249 Korean patients with chronic hepatitis B and cirrhosis, Hann et al. *(60)* observed that chronic hepatitis B males with sustained serum

Table 5
Risk Factors of HCC among HBV Carriers

Cirrhosis
Male sex
Age >40 years
Asian background
Serum ferritin >300 ng/ml
Chronic hepatitis
IgM Anti-HBc
Alcoholism

ferritin >300 ng/ml had 50% chance of developing HCC compared with 20% risk for HCC for those with lower serum ferritin levels. Further studies by the same author's group clearly demonstrated the tumor-enhancing effects of iron *(62–65)*. An increased level of immunoglobulin M antibody to hepatitis B core antigen (IgM anti-HBc) was also associated with the development of HCC *(66)*.

In order to detect HCC early among the HBsAg (+) carriers, clinicians may need to keep these risk factors in mind during the follow-up.

5.4. Secondary Prevention of HCC by Anti-HBV Therapy

During the last decade, we have witnessed great strides in the treatment of HBV infection. Six highly effective anti-HBV agents are currently available in the United States, and more agents are on the horizon (Table 6).

Table 6
Antiviral Agents Currently in Use for HBV Therapy in the United States

	FDA Approval
Interferon alpha	1992
Lamivudine	1998
Adefovir Dipivoxil	2002
Entecavir	2005
Pegasys (pegylated alpha interferon 2a)	2005
Telbivudine	2006
Tenofovir Disoproxil Fumarate	2008 (expected)
Clevudine	In Phase III trial
Emtricitabine	In Phase III trial

Prospective and retrospective studies of large numbers of chronic hepatitis B (CHB) patients with advanced liver disease including cirrhosis have demonstrated that the treatment with lamivudine (LVD) not only delays the disease progression but also reduces the incidence of HCC. In a prospective study by Liaw et al., 651 patients with CHB with fibrosis and cirrhosis were randomized to receive an antiviral agent, LVD, or placebo (2:1). Within 3 years, treatment with LVD not only delayed the disease progression but also reduced the incidence of HCC *(67)*. The incidence of HCC correlated with serum HBV DNA level at entry in a dose–response relationship. These pivotal studies re-emphasize the need for an active anti-HBV therapy for CHB patients with viral replication as the ultimate prevention and/or delay for the development of HCC.

Matsumoto et al. *(68)* in a retrospective study of 2,795 patients with CHB investigated to determine the effectiveness of LVD in preventing HCC. Of the 2,795 patients, 657 received LVD and the remaining 2,138 did not. The mean follow-up period was 2.7 years for the LVD group and 5.3 years for the non-LVD group. Annual incidence of HCC for the LVD was 0.4%/patient/year and of the non-LVD group was 2.5%/patient/year ($p < 0.001$). Recently Eun et al. *(69)* conducted a retrospective case–control study of 946 patients with CHB, who were treated from January 1983 to December 2003. Of these, 561 patients were treated with LVD and 385 did not receive LVD. The cumulative incidence of HCC for 561 LVD-treated patients was 3.3% at 2.9 years and for the 385 untreated patients the incidence was 11.2% at 3.3 years ($p < 0.001$). Recently in Taiwan, Chen et al. *(70)* conducted a large-scale longitudinal study of 3,653 HBV carriers. During the 12-year follow-up period, 164 persons developed HCC. Their extensive analysis led to the conclusion that the most important risk factor for the development of HCC is an increased serum level of HBV DNA >10,000 copies/ml regardless of the HBeAg status, alanine aminotransferase (ALT) levels, or presence of cirrhosis. The incidence of HCC correlated with serum HBV DNA level at entry in a dose–response relationship ranging from 108/100,000 person-years for an HBV DNA level of <300 copies/ml to 1152/100,000 person-years for an HBV DNA level of ≥1,000,000 copies/ml.

Undoubtedly, an active antiviral treatment for CHB patients is important to prevent the progression to cirrhosis and the development of HCC.

5.5. Antiviral Therapy for HBV

When patients with CHB develop HCC, they undergo effective local therapy for HCC. Typical treatment modalities for HCC include surgical resection, transarterial chemo-embolization (TACE), percutaneous ethanol injection (PCEI), cryoablation, or radiofrequency tumor ablation (RFA). However, without elimination of the virus, new HCC will develop in one or more sites in the liver or HCC may recur at the site of treatment. Even

after successful surgical or interventional therapy, nearly all such patients eventually die of multi-focal intrahepatic HCCs and/or of metastasis due to uncontrolled HBV replication.

With the arrival of the first oral antiviral agent, LVD, followed by adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (TLV), and tenofovir disoproxil fumarate (TDF), the survival rate of patients with HCC, including those with untreated CHB who arrive with small HCCs, has significantly improved as reported by Piao et al. *(71)* and Kuzuya et al. *(72)*. Although these authors attributed the longer survival of LVD-treated HCC patients to improvement of liver function, one could argue that the improvement of underlying liver disease including reduced recurrent or new HCC may also have resulted from suppression of HBV replication. Recent experiences of many such cases clearly support this proposition (Hann et al. personal communication). Kubo et al. *(73)* studied risk factors for recurrence after resection of HBV-associated HCC in 40 patients who underwent surgical resection. They found that high viral load was one of the risk factor for recurrence after surgery. Anti-HBV treatment for HCC patients before and after interventional therapies seems to be beneficial, although more prospective randomized studies of a large scale are required.

5.6. Anti-HBV Drugs Approved by FDA in the United States

Table 6 shows the six anti-HBV agents available currently: two interferon drugs, Interferon-α 2b and pegylated interferon-α 2a (Pegasys), and nucleos(t)ide analogues, Lamivudine, Adefovir Dipivoxil, Entecavir, and Telbivudine. Tenofovir disoproxil fumarate was approved in August of 2008. In addition, several agents such as emtricitabine, clevudine, and valtorcitabine are also in clinical trials.

6. SUMMARY

For HBV-related HCC, primary prevention of HCC is vaccination for all the uninfected individuals. Secondary prevention of HCC should focus on those who are already infected. By careful monitoring and discreet antiviral therapy with available antiviral agents we should strive to prevent the development of HCC by suppression of viral replication, elimination of the virus, and thereby delay/prevent the development of HCC in HBV-infected patients.

7. MOLECULAR BIOLOGY OF HCC ASSOCIATED WITH HBV

HCC has a number of characteristics that make it difficult to treat with current approaches. HCC is often clinically detected late, when tumors are large and in some cases have already metastasized. This is, in part, because

the liver has a large regenerative capacity and maintains homeostasis until most of the liver mass is replaced by fibrotic tissue and/or tumor. In addition, HCC often displays multidrug resistance, which may help to explain why so many of the clinical trials using standard cytotoxic drug therapy have been disappointing *(74–77)*. Hence, a number of laboratories have made efforts to dissect and understand the pathogenesis of HCC in the hope of identifying molecules that may have diagnostic/prognostic value and may be therapeutic targets that would be specific to this tumor type. Early detection of HCC would permit the use of curative therapies, such as surgical resection, liver transplantation, as well as localized chemotherapy, radiofrequency ablation, and other current treatments to be more effective *(74, 78, 79)*.

Given that HCC is among the top five tumor types worldwide *(80)*, and its overall incidence is increasing *(81)*, there is a need to understand the molecular basis of disease in order to find specific and reliable markers or early detection and to identify pathways that are specific and prevalent for this cancer. Early studies showed that the HBV carrier state and chronic liver disease (CLD) were major risk factors for the development of HCC *(20, 82)*. Further work in transgenic mice showed that overproduction and intrahepatic accumulation of HBsAg caused massive hepatocellular necrosis, triggering strong and persistent immune responses against damaged hepatocytes, resulting in rapid and persistent hepatocellular regeneration, which eventually developed into HCC *(83)*. In human carriers, there is little evidence suggesting that chronic liver disease is due to massive overproduction of virus antigens, especially in light of the facts that HBV is essentially noncytopathic and that CLD is immune mediated *(84)*. Thus, even though the pathogenesis of HCC in the HBsAg overproducing transgenic mice was different than that in human carriers, it did underscore that severe, ongoing inflammatory responses coupled with ongoing hepatocellular turnover contributed importantly to the development of cancer. The latter observations confirm that HCC is a cancer associated with chronic inflammation in the liver.

HBV encodes a small polypeptide of approximately 17 kDa, referred to as hepatitis B x antigen (HBxAg), that appears to be a promiscuous *trans*-activator *(44)*. There is accumulating evidence that HBxAg is very important in supporting virus replication and in the pathogenesis of CLD and HCC *(85)*. With regard to virus replication, HBxAg *trans*-activates the virus, thereby promoting high levels of virus replication that are often observed for years up to decades among chronic carriers *(85)*. The recent observation that sustained virus replication among carriers is a risk factor for the development of HCC *(86)* highlights one way that HBxAg contributes to tumor development. As outlined below, the fact that HBxAg is prevalent in the livers of patients with CLD *(87)*, even after seroconversion from HBeAg to anti-HBe, the latter of which is characterized by little or no virus replication, suggests that HBxAg may make other important contributions to hepatocarcinogenesis.

During chronic infection, fragments of HBV DNA integrate into the human genome at a variety of sites in practically all of the chromosomes examined *(88, 89)*. Most of these fragments span the HBx gene of HBV, and further work has shown that these fragments encode functional HBxAg in *trans*-activation assays *(90, 91)*. HBxAg is also expressed in chronically infected human livers and in HCC, where its expression levels correlate with the intensity of CLD *(87, 92, 93)*. This correlation suggests that HBxAg may protect infected hepatocytes from immune-mediated killing. Importantly, when natural effectors (or targets) of HBxAg *trans*-activation were identified, one upregulated uncharacterized gene, referred to as upregulated gene, clone 7 (URG7) conferred resistance to Fas and tumor necrosis factor alpha (TNFα)-mediated apoptosis *(94, 95)*. Further analysis showed that URG7 blocked apoptotic signals at the level of caspase 8, which is shared by Fas and TNF α signaling pathways. URG7 also stimulated phosphatidylinositol 3-kinase (PI3K) signaling, resulting in inactivation of glycogen synthase kinase 3 beta (GSK3β) and constitutive stabilization of β-catenin *(95)*. Importantly, both URG7 and β-catenin expression were upregulated in chronically infected liver tissue, especially in cells adjacent to tumor nodules, suggesting that resistance to immune-mediated apoptosis is an important step in promoting chronic virus infection and in the development of CLD.

A salient aspect of CLD is the development of fibrosis and then cirrhosis. While stellate cell activation is central to the observed changes in extracellular matrix (ECM) composition and stability, there is increasing evidence that HBxAg-expressing hepatocytes also contribute to the appearance and progression of these lesions. For example, HBxAg has been shown to upregulate the expression of transforming growth factor beta 1 (TGFβ1) in HBx transgenic mice *(97)* and in liver cell cultures stably transfected with HBx *(96)*. In the normal liver, TGFβ1 signals through a group of proteins known as Smads, which negatively regulate hepatocellular growth and maintain homeostasis *(98)*. In the presence of HBxAg, however, Smad signaling is altered so that instead of negatively regulating growth, signaling is altered to stimulate growth *(99)*. In addition to altering Smad signaling directly, HBxAg activates other signaling molecules, such as NF-kβ, PI3K, AP-1, and ras/raf/MAPK, among others *(100, 101)* that override negative growth regulation. The net outcome of these changes in signaling result in increased cell migration in vitro (which correlates with metastasis in vivo), increased ECM production, increased angiogenesis, and the development of multidrug resistance *(98)*. This may help to explain why HCC is resistant to systemic cytotoxic therapies. Importantly, upregulated expression of TFGβ1, in turn, stimulates expression of platelet-derived growth factor (PDGF), the latter of which constitutively activates β-catenin, which may act as an oncoprotein *(102)*. In related observations, overexpression of PDGF in a transgenic mouse model resulted in the development of fibrosis, steatosis, and HCC

(103) with characteristics similar to those observed in human infections. HBxAg also activates expression of fibronectin *(104)* and lysyl hydroxylase (unpublished data), the latter of which stabilizes collagen by chemical cross-linking. Further, HBxAg upregulates the strongly profibrogenic TGFβ1 by transcriptional activation and by downregulated expression of the natural TGFβ1 inhibitor, alpha 2-macroglobulin *(96)*. Although it is not known how much HBx contributes to fibrogenesis compared to activated stellate cells, it seems clear that HBV contributes to the development and progression of lesions in the liver that precede the development of HCC, which in some cases has been shown to appear within cirrhotic nodules. There is also evidence that HBxAg modulates the integrity of ECM by stimulating expression of selected matrix metalloproteinases and tissue inhibitors of metalloproteinases that are capable of breaking down ECM, thereby promoting metastasis during tumor progression *(105–109)*.

HCC, like other tumors, is an acquired genetic disease, suggesting that mutations inactivating tumor suppressor pathway components, activating oncogenes, and/or blocking DNA repair may be targets for HBxAg. In fact, there is considerable evidence that this is exactly what occurs. For example, HBxAg binds to and functionally inactivates the tumor suppressor and senescence factor p53 *(110–112)*. In this context, chronic virus infection is quite stressful to the infected hepatocyte in at least two different ways. First, HBxAg, especially associated with mitochondria, alters respiration, thereby creating an excess of oxygen free radicals *(113)* and an oxidative environment. Second, an oxidative environment in the liver is created by cell-mediated immune responses, including cytotoxic cytokine responses *(84)*. Since this environment results in oxidative damage to macromolecules, including DNA, p53 is often triggered, which blocks cell cycle progression so that mutations in the DNA could be repaired *(114)*. This process is inhibited by HBxAg, which blocks p53-dependent transcription coupled repair *(111)*, nucleotide excision repair *(115)*, and sensitizes liver cells to the effects of ultraviolet radiation *(116)*. The net effect of all this is the accumulation of mutations, most of which trigger apoptosis, but some of which get propagated and contribute to tumorigenesis. For example, inactivating point mutations of p53 are common in HCC, as well as activating point mutations in β-catenin *(117–120)*, the latter of which then acts as an oncogene in HCC. Other genes are also constitutively activated, such as those encoding c-myc *(121)*, cyclin D1 *(122, 123)*, and newly characterized proteins, such as URG4 *(124)* and URG11 *(125)*, all of which appear to contribute importantly to multi-step hepatocarcinogenesis.

HBxAg also inactivates the retinoblastoma gene product (Rb) by promoting its phosphorylation, which releases the Rb-binding protein, E2F1, a transcription factor that stimulates entry of cells into the S phase of the cell cycle *(126)*. HBxAg also downregulates transcription of $p21^{WAF1/SDI1/CIP1}$ *(127,*

128), a senescence factor that also inhibits cell cycle progression. In addition, HBxAg binds to and inactivates another senescence factor, referred to as $p55^{sen}$ *(129)*, which is overexpressed in fibroblasts from Werner's syndrome *(130)*, but also acts as a tumor suppressor when introduced into hepatoma cells *(129)*. More recently, HBxAg has been shown to overcome ras oncogene-induced senescence *(131)*. In this case, the inappropriate stimulation of oncogene expression by HBxAg triggers a compensatory negative growth regulatory response in the cell, resulting in oncogene-induced senescence. This scenario is likely to occur in fully differentiated hepatocytes early on during chronic infection, characterized by low concentrations of intrahepatic HBxAg *(87)*, but in cirrhotic nodules, where hepatocytes have already accumulated mutations in key tumor suppressor and senescence pathways and/or these pathways are inactivated in the presence of relatively high concentrations of HBxAg, senescence is finally overcome, and tumor nodules appear.

Interestingly, HBxAg activates expression of DNA methyltransferase 1 (DNMT1), in addition to other DNMTs, resulting in altered DNA methylation patterns in chronically infected liver and in HCC *(132)*. In this context, HBxAg activation of DNMT1 has been shown to promote hypermethylation of the promoter encoding E-cadherin, effectively suppressing E-cadherin expression *(133, 134)*. Since E-cadherin is an important cell adhesion molecule, loss of E-cadherin resulted in enhanced cell migration in vitro and enhanced metastasis in vivo, thereby promoting tumor progression. Both hypermethylation of presumably tumor suppressor genes and hypomethylation of presumably other growth regulatory genes have been reported in HCC *(132, 135)*, suggesting that HBxAg contributes to hepatocarcinogenesis, in part, by altered methylation (and subsequent altered expression) of many cellular genes.

The fact that HBxAg is important for virus replication, contributes to the pathogenesis of CLD, and is important to the development of HCC suggests that HBxAg would potentially be an important new target for drug discovery. Consequently, it is expected that intervention strategies that target HBxAg would be useful at most times during chronic infection. Early on in chronic infection, it is expected that targeting HBxAg would be effective against virus replication, and if so, would provide a welcome complement to the increasing number of nucleoside and nucleotide analogs that only target the polymerase activity by acting as chain terminators in the virus life cycle. It is likely that the ability to successfully target HBxAg would permit the development of combination therapy against different steps in the virus life cycle. Together with sorafenib, which attenuates the activities of several tyrosine kinases *(136, 137)*, and other compounds, which disrupt HBV nucleocapsid formation *(138),* the ability to identify new virus gene products that could be therapeutically targeted could revolutionize treatment

for HBV as combination (HAART) therapy became crucial in controlling another chronic pathogen, human immunodeficiency virus (HIV). If, as widely assumed, HBxAg *trans*-activation function is important for supporting virus replication during chronic infection and that this same function is also important in altering host gene expression that contributes importantly to the pathogenesis of CLD and HCC, then therapeutically targeting one or more of the natural effectors of HBxAg in the liver may well have an impact upon virus replication as well as pathogenesis. This would be especially true if some of the natural effectors of HBxAg that supported virus replication and gene expression on the one hand also promoted resistance to apoptosis, inactivation of tumor suppressor, senescence pathways, etc., on the other. In addition, given that HBV replicates through a pregenomic RNA intermediate by the virus-encoded reverse transcriptase, which lacks proofreading capability, means that even potent drugs targeting different virus gene products may select for resistance over time. This has already been observed with several nucleoside/nucleotide analogs *(139, 140)*. However, the mutation rates of host proteins are much lower, suggesting that the development of drugs to molecules and pathways that are essential to virus replication and pathogenesis would likely contribute to the control of chronic infection for a long time. Hence, the goal of elucidating the biology of HBxAg in HBV infection will provide multiple opportunities for the discovery and development of new types of drugs directed specifically against virus gene products as well as pathways that contribute to CLD and HCC.

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8 Hepatitis C and Hepatocellular Carcinoma

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CONTENTS

Abbreviations

HCC	hepatocellular carcinoma
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
IFN	interferon
UTRs	untranslated lesions
ALT	alanine aminotransferase
NS	nonstructural
US	ultrasonography

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CT	computed tomography
NASH	non-alcoholic steatohepatitis
AST	aspartate aminotransferase
MDCT	multi-detector computed tomography
AFP	alpha-fetoprotein
HGF	hepatocyte growth factor
IGF-1	insulin-like growth factor
SUV	standardized uptake value
QALY	quality-adjusted life-year
DCP	des-gamma-carboxyprothrombin

ABSTRACT

HCC usually develops in patients with chronic liver disease, those accompanied by cirrhosis in particular. Ultrasonography and tumor marker tests play important roles in HCC surveillance in patients with chronic liver disease and are widely used. The primary prevention of HCV-related HCC includes strategies for the prevention of HCV infection and for viral eradication. Regarding the former, novel HCV transmission in the general population has been declining in many countries, as evidenced by the lower prevalence of HCV infection among younger generations. The effect of IFN therapy on the prevention of HCC remains controversial. The possibility of curative treatment depends on both tumor stage and liver function. Effective treatments for HCC include percutaneous ablation, surgical resection, and liver transplantation. Both percutaneous ablation and surgical resection provide a high rate of complete responses and are assumed to improve survival that should exceed 50% at 5 years. Liver transplantation shows a better survival rate and is not contraindicated by advanced liver dysfunction. However, its application is limited by the scarcity of donor organs. Although short-term prognosis of HCC patients has been much improved recently due to advances in early diagnosis and treatment, long-term prognosis is as yet far from satisfactory due to frequent recurrence. Prevention of recurrence of HCC remains one of the most challenging tasks in current hepatology.

Key Words: Chronic hepatitis C; Hepatocellular carcinoma; Epidemiology; Pathology; Surveillance; Ultrasonography; Tumor marker; Radiofrequency ablation; Resection; Transplantation; Tertiary prevention

1. INTRODUCTION

Hepatocellular carcinoma (HCC), one of the most common cancers worldwide *(1)*, usually develops in a liver already chronically damaged,

often from cirrhosis. The etiology of liver disease, and consequently that of HCC, differs geographically. In most areas, chronic viral hepatitis due to either hepatitis B virus (HBV) or hepatitis C virus (HCV) is the main cause of HCC *(2–5)*. In this chapter we focus on HCC among patients with hepatitis C.

2. EPIDEMIOLOGY

HCV infection has shown rapid worldwide expansion in recent years *(6)*. HCV is transmitted as a blood-borne infection, although it is much less infectious than HBV (Table 1). Mother–neonate transmission and horizontal sexual transmission are uncommon with HCV. Therefore, the recent rapid spread of HCV must be associated with some artificial change in the environment. Epidemiological studies have shown that viral spread began in the United States in the mid-1960s, mainly among intravenous drug users, and then began to decline by the 1990s, when general concern regarding human immunodeficiency virus (HIV) infection increased substantially. Indeed, in the United States, the transmission route of HCV overlapped that of HIV. This led to a serious medical problem, HCV/HIV co-infection, in which liver damage progresses more rapidly due to comorbid immunosuppression. Currently, approximately one-tenth of all patients with HCV infection in the United States are also infected with HIV. With improved treatment for HIV, HCV-related disease is currently the primary cause of mortality in patients with HIV/HCV co-infection *(7)*. In contrast, in Egypt, where the estimated prevalence of HCV infection is 10% or higher, the virus is thought to be transmitted via a peculiar iatrogenic route due to parenteral antischistosomal therapy using serum from infected donors, which was widely practiced from the 1960s to the early 1980s *(8)*. This resulted in the predominance of HCV genotype 4a, which is unique to Egypt.

Table 1
Epidemiology of Chronic HBV or HCV Infection in Japan

Virus	*HBV*	*HCV*
Vertical transmission	Common until early 1980s	Rare
Horizontal transmission	Rare in adulthood	Common until 1990 (Peaked in 1950s–1960s)
Prevalence	0.8%	1.5–2.0%
Etiology in HCC	10–15%	75–80%

In Japan, HCC-related mortality has more than tripled since the mid-1970s. The emerging cases of HCC were typically negative for HBV and developed in patients with so-called non-A, -B hepatitis, which was later revealed to be almost entirely equal to chronic hepatitis C *(9)*. Presently, HCV infection is responsible for 75–80% of the cases of HCC in Japan, while HBV is responsible for 10–15% *(10)*. About 40% of HCV-related HCC patients in Japan have a history of blood transfusion, typically within the 1950s and the 1960s. At that time, the supply of blood for transfusion in Japan was dependent on paid blood donors, many of whom were also intravenous drug users, mainly methamphetamine, among whom HCV is thought to have spread first in Japan after the end of World War II. In addition, the routine reuse of syringes and needles in medical practice at that time may have contributed to further viral spread. Commercial blood banks were abolished by 1969 in Japan and replaced by the Japanese Red Cross Society, which is fully dependent on voluntary blood donation. Syringe and needle reuse were also strongly discouraged in the 1970s. Consequently, viral spread in Japan began to decline in the 1970s, although HCV transmission through blood transfusion continued until the advent of a sensitive HCV detection system in the early 1990s. In Japan, there was an interval of at least 30 years between peak HCV spread and peak incidence of HCV-related HCC. Considering the interval of 20 years between the peak viral spread in Japan and the United States and the fact that it takes 20 years or longer from HCV infection to HCC development, a further increase in the incidence of HCC in the United States appears to be inevitable *(11, 12)*.

Subtyping HCV has been important for at least two major reasons in clinical practice: from an epidemiological perspective and because of the predictive value in interferon (IFN) therapy. Epidemiological studies have revealed the geographical distribution of HCV genotypes worldwide *(13)*. From a clinical viewpoint, subtyping HCV is very useful for predicting the likelihood of a treatment response and, in many cases, determining the duration of treatment *(14–16)*. In addition, there are several reports that genotype 1b is associated with an increased cytopathic effect. According to Silini et al. *(17)* HCV genotype 1b infection is very rarely found in patients with minimal chronic liver disease, which is associated with persistently normal alanine aminotransferase (ALT) and slow disease progression. Feray et al. *(18)* reported that the recurrence of hepatitis with genotype 1b after liver transplantation was more severe and progressive than for other genotypes.

3. PATHOLOGY

HCV, a positive-stranded RNA virus, is a major causative agent of HCC worldwide. However, the molecular mechanisms of HCV-induced hepatocarcinogenesis remain unclear. HCV is distantly related to the flaviviruses

and pestiviruses of family Flaviviridae. There have been no reports that flaviviruses or pestiviruses are integrated into the human genome, so it may be impossible for HCV to exert its oncogenecity through integration into the host genome. HCV has an approximately 10-kb genome containing a large open reading frame encoding a polyprotein precursor of around 3,000 amino acids and untranslated regions (UTRs) at the 5′- and 3′-ends of the genome (Fig. 1). The putative organization of the HCV genome includes (from the 5′- to 3′-end), the 5′-UTR, three or four structural proteins (core, E1, E2/p7), six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and the 3′-UTR *(19–21)*. It is thought that continuous inflammation, apoptosis or necrosis, and hepatocyte regeneration caused by HCV infection may increase the chance of gene alteration and cause hepatocarcinogenesis. However, accumulated data suggest that HCV proteins are directly involved in regulating hepatocyte proliferation. In fact, HCV proteins have various functions other than HCV replication in host cells, some of which may be directly or indirectly related to hepatocarcinogenesis (Table 2) *(22)*.

Recently, it was shown that HCV infection enhances DNA damage and the mutation of cellular genes, including proto-oncogenes *(23–25)*. In addition, the expression of the core protein impairs DNA repair in human

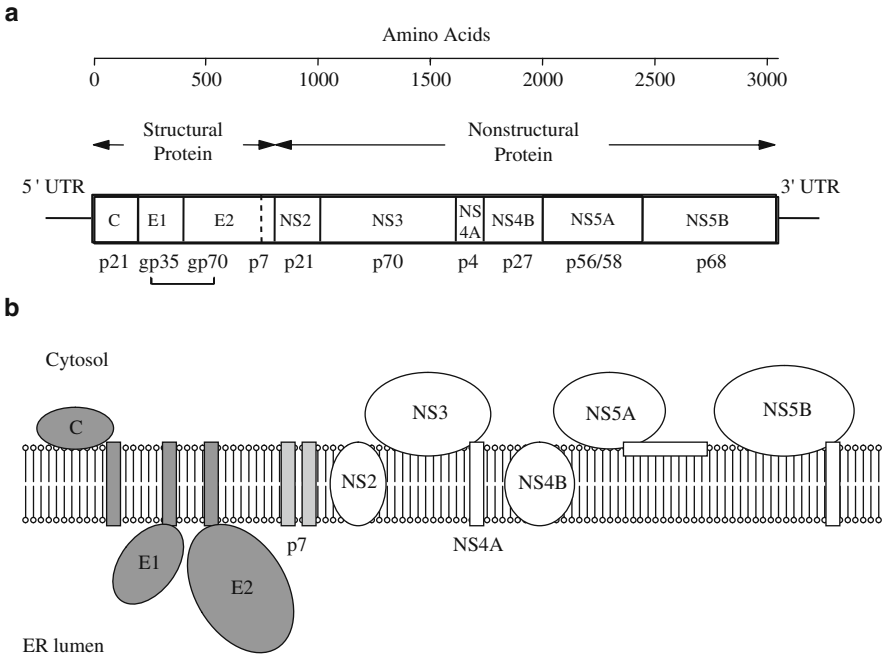

Fig. 1. Structure of hepatitis C virus.

Table 2
Function and Oncogenic Potentials of Proteins

Protein	*Function*	*Oncogenic potentials*
Core	Nucleocapsid	Cell transformation Carcinogenesis in transgenic mice Transcriptional regulator Anti-apoptosis Activation of proto-oncogenes Repression of tumor suppressor genes Impairment of DNA repair
E1	Envelope	Unknown
E2	Envelope	Unknown
P7	Ion channel	Unknown
NS2	Metalloprotease	Unknown
NS3	Serine protease Helicase	Cell transformation Anti-apoptosis Repression of tumor suppressor genes
NS4A	Serine protease cofactor	Unknown
NS4B	Unknown	Cell transformation
NS5A	Unknown	Cell transformation Anti-apoptosis Repression of tumor suppressor genes Induction of chromosome instability
NS5B	RNA-dependent RNA polymerase	Repression of tumor suppressor genes

hepatoma cells *(26)*. The resulting accumulation of mutations in cellular genes may lead to cell transformation. Moreover, iron overload is reported to induce mitochondrial injury and increase the risk of HCC development in transgenic mice expressing HCV polyprotein *(27)*.

HCV proteins regulate the transcription of cellular genes, including p53 and p21, activate signal transduction pathways, and suppress apoptosis. These functions of HCV proteins may lead to hepatocyte proliferation and transformation. To clarify the molecular mechanisms of HCV-induced hepatocarcinogenesis, comprehensive functional analyses of HCV proteins are needed. The recently developed HCV subgenomic replicon *(28)* and robust HCV infection systems *(29–31)* will facilitate analyses of the effect of not only HCV proteins but also HCV replication.

4. PRIMARY PREVENTION OF HCC

HCC is a unique malignancy in that known acquired factors (i.e., chronic viral hepatitis B and C) are the predominant causes of carcinogenesis, which is of enormous clinical importance *(32, 33)*. By screening for HBV/HCV infection, we can identify patients at high risk of HCC and perform cost-effective surveillance. Screening policies should be based on the prevalence of each viral infection in specific geographic areas. This will result in the secondary prevention of HCC through early detection and treatment. Furthermore, the primary prevention of HCC (i.e., reducing its risk factors) is possible by controlling virus infection. In fact, HBV vaccination has been shown to be effective in decreasing HBV-related HCC and the awareness of the control of blood-borne infection in both the medical practice and the general population has apparently curbed further propagation of HCV infection. Antiviral therapy for patients already infected is another aspect of primary prevention.

The primary prevention of HCV-related HCC includes strategies for the prevention of HCV infection and for viral eradication. Regarding the former, novel HCV transmission in the general population has been declining in many countries, as evidenced by the lower prevalence of HCV infection among younger generations. Viral transmission through blood transfusion can be prevented by screening donor blood using sensitive assays. Although campaigns against blood-borne viral transmission, including both HCV and HIV, should be sustained vigorously, effort can now be focused on viral eradication in patients who have already been infected with HCV.

The effect of IFN therapy on the prevention of HCC is controversial. Studies performed in the United States have failed to show a reduction in the incidence of HCC after IFN therapy. In contrast, many clinical studies performed in Japan have clearly demonstrated that the incidence of HCC was reduced among IFN-treated patients showing a sustained virologic response *(34, 35)*. The resolution of cirrhosis was also noted following a sustained virologic response *(36)*. These beneficial effects are expected to be enhanced by the advent of combined PEG-IFN and ribavirin therapy *(14, 15)*. The discrepancy in the preventive effect of IFN therapy on HCC between Japanese and American studies may result from different patient characteristics, such as the ages of HCV-infected patients; further investigation is required.

5. SURVEILLANCE

Ultrasonography (US) and tumor marker tests play important roles in HCC surveillance in patients with chronic liver disease and are widely used. However, there is insufficient evidence to suggest that such surveillance improves the prognosis of patients with HCC or increases the effectiveness

of local therapies, such as resection and local ablation therapy, or indeed radical treatments, such as liver transplantation. Similarly, the usefulness of computed tomography (CT) or magnetic resonance imaging (MRI) in HCC surveillance remains unclear.

The primary objective of screening and HCC surveillance should be to reduce mortality as much as possible in patients who actually develop cancer, in an acceptable, cost-effective fashion. To attain this objective, two distinct issues deserve meticulous consideration: the target population and mode of surveillance.

5.1. Target Population

HCC shows significant regional clustering *(4)*. HBV, HCV, and other environmental factors may play important roles in the development of HCC, with the relative importance of individual factors varying widely according to geographic area *(3*, *5*, *37*, *38)*. In Japan, HCV infection is responsible for about 80% of the cases of HCC, whereas HBV infection is responsible for 10% and alcohol for about 5% *(39*, *40)*. These values may differ substantially in other countries. For example, in China, where the prevalence of HBV infection is much higher, HBV infection is by far the predominant etiologic factor for HCC. In the United States, non-alcoholic steatohepatitis (NASH) is reportedly a major factor in HCC.

Given the low incidence of HCC in individuals without risk factors, surveillance is not recommended for the general population. A commonly accepted rate that requires surveillance is greater than 0.2% per year. Therefore, the first step in screening for HCC is to screen patients at risk of developing HCC. Because chronic viral hepatitis due to either HBV or HCV may be asymptomatic, mass screening for hepatitis virus infection, either HBV or HCV, is justified if the prevalence of infection is reasonably high in a region. Indeed, in Japan, the general population over 40 years of age has undergone mass screening for HBV and HCV infection since 2002, although the cost-effectiveness of this program remains to be evaluated.

Persistent HBV infection is a major risk factor for HCC. HBV carriers have a 223-fold higher risk of developing HCC than non-carriers *(41)*. Among HBV carriers, HBe antigen-positive patients are at a higher risk of HCC than HBe antigen-negative patients (relative risk, 6.3-fold) *(42*, *43)*. Recently, the results of a large-scale, long-term cohort study conducted in Taiwan showed that the serum HBV DNA level is the strongest risk factor for both the progression to cirrhosis and the development of HCC among HBV-positive patients, independently of serum HBe antigen/antibody status or ALT levels *(44)*. Together with the advent of reliable quantitative assays, the determination of HBV DNA levels may replace the determination of HBe antigen/antibody status as a risk indicator for HCC.

While the prevalence of chronic HBV infection is high in some geographic areas, such as East and Southeast Asia and sub-Saharan Africa, the prevalence of chronic HCV infection has recently increased in some developed countries, including Japan, southern European countries, and the United States. In chronic hepatitis C patients, the risk of developing HCC increases with the progression of liver fibrosis (Table 3) *(34*, *45)*, and chronic hepatitis C patients with cirrhosis have a very high risk of HCC *(46)*. In European countries and United States, annual incidence rate of HCC is reported to be 0.5–5% *(47)*. The reason of this difference is not well known, but maybe related to the difference in the age of patients. Ethnic difference may also be involved. In Japan, HCV infection spreads nationally mainly in the 1950s and in the 1960s and is currently, after several decades required for progression to cirrhosis, the predominant cause of HCC. Peak viral spread in the United States occurred two decades later, and the incidence of HCV-related HCC is now increasing rapidly *(2*, *48)*. In addition to the degree of liver fibrosis, male gender, older age, and heavy alcohol consumption are known risk factors for HCV-related HCC.

Table 3
Incidence of HCC According to Histological Fibrosis Stage Reported from Japan

Fibrosis stage	*Annual Incidence of HCC*	*Risk Ratio (95% CI)*
F0/1	0.5% (3/160)	1
F2	2.0% (11/164)	4.431 (1.704–11.522)
F3	5.3% (13/59)	13.097 (5.194–33.021)
F4	7.9% (32/107)	24.011 (9.638–59.815)

Cirrhosis due to etiologies other than chronic viral hepatitis also confers a risk of developing HCC. Major etiologies include alcoholic liver disease and NASH *(49–51)*, whose relative importance may differ geographically. Hassan et al. *(52)* reported that alcoholic liver disease accounted for 32% of all HCC cases in an Austrian cohort. In the United States, the approximate annual hospitalization rate for HCC related to alcoholic cirrhosis is 8–9/100,000 compared to approximately 7/100,000 for hepatitis C *(53)*. NASH is a chronic liver disease that is gaining increasing significance due to its high prevalence worldwide and its potential progression to cirrhosis, HCC, and liver failure. Although NASH has been described in cohorts of HCC patients *(54*, *55)*, the incidence of HCC in cirrhosis due to NASH is unclear. Aflatoxin may play a role in certain areas.

In brief, the evaluation of the degree of liver fibrosis is of paramount importance in assessing the risk of HCC in patients with chronic liver disease of any etiology. Histologic evaluation of liver biopsy samples has been

considered the gold standard for assessing liver fibrosis. However, the invasiveness of a liver biopsy limits its clinical feasibility. In clinical practice, repeated assessment of liver fibrosis is often required because a non-cirrhotic liver may become cirrhotic over time, sometimes rather rapidly. Consequently, the non-invasive evaluation of liver fibrosis is one of the main areas of interest in hepatology.

One such non-invasive method, transient elastography, correlates well with the histological stage of liver fibrosis *(56–60)*. The reported cutoff value for the diagnosis of histological cirrhosis was 12.5–14.9 kPa. Higher values of liver stiffness may require proper attention regarding decompensation and HCC development *(61)*. The FibroTest is based on the age and gender of patients combined with five biochemical markers (total bilirubin, haptoglobin, γ-glutamyl transpeptidase, alpha-2 macroglobulin, and apolipoprotein A1) *(62)*. An index of 0–0.10 had a 100% negative predictive value, while an index of 0.60–1.00 had a greater than 90% positive predictive value for a Metavir score of F2 to F4. APRI is the aspartate aminotransferase (AST) level/upper limit of normal divided by the platelet count (10^9/L) multiplied by 100 *(63)*. For a hypothetical patient with an AST of 90 IU/L (upper limit of normal 45) and a platelet count of 100 ($\times 10^9$/L), the APRI is 2.0, which means the patient has a 41% likelihood of advanced fibrosis and 5% chance of having minimal or no fibrosis. The applicability of these methods in surveillance requires evaluation in future prospective studies.

Patients who are considered to be at a non-negligible risk of HCC development should be subjected to a surveillance program, as discussed below. Possible exceptions may include those with severe liver dysfunction who would not receive any treatment if diagnosed with HCC or those with other life-threatening illnesses.

5.2. Surveillance Methodology

Traditionally, two methodologies have been used for HCC surveillance in high-risk patients: tumor marker determination and diagnostic imaging. Serum alpha-fetoprotein (AFP) concentration is representative of the former and liver ultrasonography (US) of the latter. The usefulness of a surveillance program should be evaluated based on the beneficial effects on the outcome of HCC patients diagnosed via these modalities relative to cost. However, few prospective randomized trials have compared the outcome of HCC patients in or outside a surveillance program. Therefore, the currently available evidence regarding the effects of surveillance on decreasing overall or disease-specific mortality has come mostly from retrospective or case–control studies.

5.2.1. AFP

AFP is a glycoprotein with a molecular weight of 72 kDa. The main physiological function of AFP appears to be the regulation of fatty acids in fetal and proliferating adult liver cells *(64)*. Since 1968, AFP has been used as a serum marker for human HCC *(65)*. As a marker, AFP reportedly has a sensitivity of 39–65%, a specificity of 76–94%, and a positive predictive value of 9–50% *(66–71)*. Studies assessing the usefulness of AFP in HCC screening have varied widely in their design and in the characteristics of targeted patients in terms of etiology, severity of background liver disease, and so forth. Moreover, specificity and sensitivity inevitably depend on the cutoff level selected for diagnosis.

An intrinsic disadvantage of AFP as a tumor marker is the fact that the serum AFP levels can increase in patients without HCC when hepatitis is active, partly due to accelerated cellular proliferation in regeneration. Because serum AFP rarely exceeds 20 ng/mL in healthy subjects, this value is often adopted as the upper limit of normal for serum AFP. However, values slightly above this level may not be indicative of HCC among patients with chronic hepatitis, whereas adopting a low cutoff value results in low specificity. AFP levels exceeding 400 ng/mL can be considered almost definitively diagnostic of HCC, but sensitivity inevitably decreases with higher cutoff levels. An additional disadvantage of AFP as a tumor marker is that small HCC tumors, the detection of which is the primary objective of surveillance, are less likely to be AFP producing, and serum AFP level may not reach the diagnostic limit even if they are AFP producing.

It has been proposed that AFP determination should be used as a screening test only when US is either unavailable or of such poor quality that lesions smaller than 2 cm in diameter will not be detected. One such case is HCC screening in Alaskan hepatitis B carriers, among whom AFP testing allowed the detection of tumors at an earlier, treatable stage *(72)*. Although the screened subjects had an increased survival compared to historic controls, this must have been affected by the lead-time and length–time bias inherent to retrospective studies on screening.

5.2.2. US

US became available for identifying intrahepatic lesions in the early 1980s *(73)*. This imaging modality is appealing because it is almost completely non-invasive. The ribs and air in the lungs and gastrointestinal tract surrounding the liver may hinder ultrasound imaging, but imaging of the liver has been facilitated by improvements in devices and techniques. The reported sensitivity of US for detecting HCC nodules is highly variable, ranging from 35 to 84% *(74)*, depending on the expertise of the operator

and the ultrasound equipment used. Indeed, more sophisticated ultrasound instruments can produce images with much better resolution, improving the detectability of small intrahepatic lesions. Note, however, that ultrasound diagnosis is heavily operator dependent. A high level of skill and experience is required to record high-quality images and make an accurate diagnosis. In addition, an ultrasound diagnosis may not be possible due to the patient's physical condition, such as severe obesity.

The reported sensitivity of US for HCC detection is as low as 20.5% *(75)* based on the pathology of explanted livers that were removed from patients who underwent liver transplantation. Small HCC nodules less than or equal to 2 cm in diameter constituted 85% of the lesions that were not detected ultrasonographically *(76)*. The ultrasound detectability of HCC nodules depends on tumor size: nodules >5.0, 3.1–5.0, 2.1–3.0, and 1.0–2.0 cm in diameter had detection rates of 92, 75, 20, and 13.6%, respectively *(75)*.

Although these data are rather disappointing, other reports indicate that the detectability of intrahepatic nodules with US is almost comparable to that of CT *(77–80)*. In a study of nodules that were $\leq$2 cm in diameter in patients with chronic hepatitis, the detection capability of US exceeded that of CT or MRI for nodular lesions, and US was superior for the detection of adenomatous hyperplasia and well-differentiated HCC *(81)*. Overall, US is indispensable in the screening of HCC, as it is non-invasive and less expensive. However, the definitive diagnosis of HCC depends on the evaluation of its vascularity, which is not possible via conventional US. Instead, CT or MRI with contrast enhancement is required when a suspected lesion is identified via US.

US, when conducted by less-experienced operators, has several shortcomings. Moreover, the resolution may not be satisfactory in cirrhosis patients with rough echo patterns in the background liver. Therefore, effective HCC detection requires combined US with CT or MRI. However, there are few reports on HCC surveillance that actually used CT or MRI, and its cost–benefit ratio remains unclear.

Recently, several contrast enhancement materials have been developed for US. These materials are very useful in the differential diagnosis of intrahepatic nodules or the demarcation of intrahepatic lesions before percutaneous ablation. However, their role in HCC screening is yet to be defined.

5.2.3. Combined AFP and US in HCC Surveillance

Although serum AFP measurement is generally less sensitive than US, their specificities may be comparable when using appropriate cutoff values. HCC screening via combined US and AFP may lead to improved detection, although previous reports have been generally negative *(67, 82–84)*.

However, in a non-randomized study of patients with cirrhosis, the sensitivity of detection was reported to be increased using both US and AFP measurements, as compared to either alone *(82)*.

Recently, a randomized trial evaluated HCC screening using AFP and US every 6 months compared to no screening in over 18,000 Chinese patients with HBV infection *(85)*. More cases of HCC were diagnosed in the screened group than in the non-screened group (86 versus 67) and overall survival was higher in the former group (65.9, 52.6, and 46.4% at 1, 3, and 5 years, respectively) than in the latter (31.2, 7.2, and 0% at 1, 3, and 5 years, respectively).

A retrospective study assessed HCC screening in 367 patients of 70 years or older, with AFP measurements and US every 6 or 12 months. The screening allowed more frequent diagnosis of HCC at an early stage, increased the proportion of patients who could receive a curative treatment, and improved their prognoses compared to unscreened patients. The apparent survival benefit was restricted to the first 3 years after the detection of HCC, probably because of the shorter life expectancy of elderly people *(86)*.

5.2.4. New Serum Markers and New Methods

Recent developments in gene expression microarrays, proteomics, and tumor immunology permit thousands of genes and proteins to be screened simultaneously. In the next decade, new biomarkers should be established for cancer screening, including HCC. To establish a formal framework to guide biomarker evaluation and development, a five-phase program was adopted by the Early Detection Research Network (EDRN) of the National Cancer Institute *(87)*. Currently, several new markers appear promising, including des-gamma-carboxyprothrombin (DCP), AFP-L3, glypican-3, insulin-like growth factor (IGF)-1, and hepatocyte growth factor (HGF). These markers are to be further evaluated in phase 2 studies to determine their ability to detect early-stage HCC, followed by phase 3 studies that will retrospectively determine whether they can detect preclinical disease. Pending these results, phase 4 studies will be performed to assess prospectively their ability to detect early HCC and phase 5 studies will be performed to confirm that surveillance using these markers reduces morbidity and mortality from HCC.

The detection sensitivities of dynamic CT and dynamic MRI are both high for hypervascular HCC. Because patients with HCC undergo repeated imaging examinations and the diagnostic capabilities of dynamic CT and MRI are similar, dynamic MRI, which does not involve exposure to X-rays, may be superior to CT. However, MRI systems that allow high-quality dynamic studies are not yet as widely used as high-speed CT systems.

Institutions without access to dynamic MRI may instead rely upon high-speed dynamic CT, such as helical CT, or even more advanced systems, such as multi-detector CT (MDCT). The development of MDCT has dramatically accelerated scan acquisition in liver CT *(88)*. With MDCT, high-speed volume coverage of the entire liver is possible in 4–10 s, which allows the acquisition of two separate series of scans in the arterial phase, termed early arterial and late arterial phase scans *(89, 90)*. With fluorodeoxyglucose positron emission tomography (FDG-PET), tumor cells with active glucose metabolism take up and specifically accumulate ^{18}F-FDG, blocking the metabolic pathway. In a study evaluating the diagnosis of HCC using a quantitative standardized uptake value (SUV), the SUV for HCC was lower than that of metastatic liver cancer *(91)*. In general, FEG-PET is not recommended for the diagnosis of HCC because it is expensive and not superior to conventional diagnostic imaging techniques, such as CT and MRI.

6. STANDARDIZED RECALL PROCEDURES

Once patients are identified via an abnormal surveillance test, they need to be recalled for subsequent evaluation. However, despite various recall algorithms described in the literature, none has been tested in a prospective fashion. Furthermore, recall procedures should differ based on abnormal AFP versus US findings. Increases in serum AFP need to be interpreted against background liver disease. Reactivated chronic hepatitis B is often accompanied by increased AFP levels. Pregnancy may cause temporary elevation of AFP levels, sometimes together with an increase in the proportion of the L3 fraction. Therefore, patients with increased serum AFP levels require a detailed clinical evaluation to determine the cause of the increase.

When a low-echoic lesion is newly detected with US in the liver of a patient at risk of HCC, a complete evaluation is required. Typically, this involves CT or MRI with contrast enhancement and the presence of hyperattenuation in the arterial phase with washout in the late phase can be considered as a definitive sign of HCC *(92)*. In ambiguous cases, a needle tumor biopsy under ultrasound guidance is recommended. However, it is controversial whether all suspicious nodules should be subjected to liver tumor biopsy because of concerns regarding potential tumor seeding.

7. SCREENING INTERVAL

Because the risk of HCC development does not usually decrease spontaneously in patients who are targets for HCC screening, an HCC surveillance program should consist of repeated screenings at a determined interval. US

is superior to CT in this regard because it is non-invasive and cost-effective. The guidelines of the American Association for the Study of Liver Diseases (AASLD) propose ultrasound surveillance for patients at high risk of HCC at an interval of 6 months. The guidelines explicitly indicate that the surveillance interval should depend not on the risk of HCC but exclusively on tumor doubling times, to detect cancer nodules while they are small enough for curative treatments.

In contrast, in Japan, ultrasound surveillance at a shorter interval of 3–4 months is encouraged for extremely high-risk patients, whereas an interval of 6 months is recommended for high-risk patients *(93)*. Chronic hepatitis C patients with cirrhosis in Japan have HCC incidence rates of 6–8% per year, constituting an extremely high-risk group. Theoretically, shorter surveillance intervals lead to tumor detection at smaller sizes. However, it is unknown whether the difference in detected tumor size, if any, is large enough to affect the prognosis in a cost-effective fashion. Although there is no prospective comparison of different schedules, one retrospective study of cirrhosis patients and a mathematical model applied to hepatitis B virus careers suggested that a longer screening interval is as effective as a 6-month interval in terms of survival.

It is controversial whether AFP determination should be included in HCC surveillance programs. However, if AFP is to be measured, it should be measured repeatedly and an abnormal AFP level must be interpreted not by simple comparison with a given cutoff value but in the context of the temporal series. An abrupt elevation of serum AFP levels in the absence of exacerbation of hepatitis may indicate the development of HCC, even if US is apparently negative, and further evaluation with contrast-enhanced CT or MRI should be considered.

8. COST-EFFECTIVENESS

According to a decision analysis model, the cost-effectiveness ratio for screening European patients with Child–Pugh class A liver disease ranged between $48,000 and $284,000 USD for each additional life-year gained *(94)*. However, this study did not consider liver transplantation as a treatment option. In a group of patients who could anticipate excellent survival, the cost-effectiveness ratio ranged between $26,000 and $55,000. In another study of 313 Italian patients with cirrhosis undergoing serum AFP analysis and liver US every 6 months, the cost per case of treatable HCC was $17,934, and the cost per year of life saved was $112,993 *(70)*. In the United States, the cost for each quality-adjusted life-year (QALY) gained through surveillance was estimated to range from $35,000 to $45,000 *(94)*. HCC screening

in patients waiting for liver transplantation has been associated with a cost per year of life saved ranging from $60,000 to $100,000, depending on the screening modality used *(95)*.

It must be emphasized that the cost-effectiveness of HCC screening has been assessed via retrospective analyses or using decision models. While retrospective studies suffer from selection bias, decision analysis models are based on a simulation of costs and health outcomes and results may vary greatly according to different assumptions, such as the incidence of HCC in the screening population, the screening interval, the modality of diagnosis, the type of treatment after diagnosis, the doubling time of tumors, and the tumor recurrence rate. In particular, there must be a feasible treatment modality that favorably affects prognosis if screening is to be cost-effective.

9. PREVENTION OF RECURRENCE

The short-term prognosis of HCC patients has greatly improved due to recent advances in early diagnosis and treatment. However, the long-term prognosis remains far from satisfactory, as indicated by the fact that the overall survival 10 years after apparently curative treatment of HCC is as low as 22–35% *(96, 97)*. In HCC patients, the slope of a typical cumulative survival curve does not level out over time after treatment. In contrast, in most other malignancies, the slope of the cumulative survival curve levels out in about 5 years after relatively curative treatment. In other words, HCC is rarely treated curatively, and the primary reason for this is the frequent recurrence of HCC, even after apparently curative treatment involving either local ablation or surgical resection *(98)*. Unlike liver transplantation, these locoregional therapies do not remove microscopic lesions in the remaining liver. However, this does not explain the fact specific to HCC that the risk of recurrence does not decline over time. In fact, recurrent HCC continues to develop at an annual rate of 10–20%. This continual recurrence of HCC after initial treatment is thought to be mostly due to multicentric de novo carcinogenesis. In this respect, liver transplantation is superior to locoregional therapy.

At least theoretically, however, strategies similar to those used in primary prevention may be applicable to HCC recurrence due to multicentric carcinogenesis. Recent antiviral therapies, in particular, may be considered in this regard. HCV-related HCC patients have undergone IFN therapy after the initial treatment, which may have reduced the incidence of recurrence *(99, 100)*. Liver function did not deteriorate in patients who achieved a sustained virologic response with IFN therapy, among whom there was no death due to liver failure. Consequently, overall survival improved in patients subjected to IFN therapy.

Needless to say, early diagnosis and complete removal of primary HCC lesions are requisite for antiviral therapy. In other cases, safe, effective chemotherapeutic agents would be useful as adjuvant therapy for relatively advanced HCC where undetectable intrahepatic metastases are suspected. However, conventional chemotherapeutic agents are not satisfactorily effective against HCC nor safe enough for protective long-term use. Hasegawa et al. *(101)* reported that the administration of uracil-tegafur (UFT) as an adjuvant chemotherapy for hepatic resection offered no evidence of potential benefit and overall survival appeared to be worse in the treatment group. The authors suggested that the adverse effects of UFT on liver function were responsible for poor survival in the treatment group. Some agents appear promising in terms of safety, but their effects remain to be confirmed *(102, 103)*. The prevention of the recurrence of HCC, or tertiary prevention, is currently one of the most challenging tasks in hepatology.

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9 Metabolic Disease and Hepatocellular Carcinoma

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CONTENTS

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ABSTRACT

The pathophysiologic mechanisms recognized as inducing changes in the cell cycle and its regulation that enables carcinogenesis to occur are presented. More importantly, the mechanisms thought to be most important in each metabolic disorder that can terminate in the development of a hepatic cancer are identified. Often more than one mechanism is involved and it is the sum mutation of these metabolic events with environmental hazards and exposures that enable a cancer to develop or not in an individual with a metabolic disease having an association with hepatic cancer.

Key Words: Free-radical injury; oxidative stress; epigenetic dysfunction; inducible genetic errors; hypo/hypermethylation; cell cycle disruption; genetic disorders

1. INTRODUCTION

Hepatocellular carcinoma is the most common cause of primary liver cancer accounting for more than 80% of cases *(1, 2)*. It is only second in frequency to all forms of metastatic cancers to the liver combined (colon, stomach, pancreas, breast, lung) as a cause of liver cancer. More than 1 million deaths each year occur as a result of hepatocellular carcinoma, and it accounts for one-third of all the cancer-related deaths occurring annually worldwide *(3, 4)*. The ratio of hepatocellular cancer deaths occurring annually to the incidence of new hepatocellular carcinomas in the population ranges between 0.85 to 0.90 and documents the severity of the disease process once identified *(5, 6)*.

The risk factors for hepatocellular carcinoma vary geographically and include cirrhosis of any cause, chronic hepatitis (especially HBV), toxin-induced liver diseases (alcohol, tobacco, aflatoxins, other chemicals and drugs), chronic viral hepatitis (HBV and HCV), and various metabolic liver diseases *(7)*. This last group is rather small but it is an important group to recognize and, as a result, to screen for the development of hepatocellular carcinoma. If identified early, liver transplantation cures not only the hepatic

cancer but also the metabolic abnormality and the cirrhosis present in these cases.

Other risk factors for hepatocellular carcinoma include male gender, increasing age at the time of HBV or HCV infection, obesity, diabetes mellitus, nonalcoholic fatty liver disease, especially nonalcoholic steatohepatitis and chronic cholestasis. Each of these factors can coexist in an individual with a metabolic liver disease and affect the disease outcome and potentially enhance the risk for hepatic cancer. Despite the impressive evidence for the prevention and control of HBV infection occurring as a consequence of childhood vaccination programs and current antiviral therapies, the incidence of hepatocellular, at least in the western world, is increasing rather than decreasing *(2–4)*. This increase in hepatocellular carcinoma cancer is primarily due to the increase in cases associated with HCV infection, nonalcoholic steatohepatitis, cryptogenic cirrhosis, obesity, and diabetes mellitus, all of which except HCV are components of the metabolic syndrome. It is interesting to consider the potential role of being heterozygous for genes associated with genetic hemochromatosis, alpha 1 antitrypsin deficiency, methylenetetrahydrofolate reduction deficiency, and other genetic disease in rare cases with a newly recognized hepatocellular carcinoma. The vast majority of such cases manifest biochemical evidence of insulin resistance which is characterized by an increased insulin level relative to the plasma glucose level or by an increased glucose level together with normal or even increased serum insulin levels. It may well be that insulin resistance per se may be the underlying factor responsible for the development of hepatocellular carcinoma in most of these cases of hepatocellular cancer. Certainly, growth factors including insulin are recognized as playing at least some role in the pathogenic mechanisms culminating in the development of hepatocellular carcinoma *(8)*. On the other hand, increased serum insulin levels can be the result of the metabolic changes taking place with in the liver.

Hepatocellular carcinomas are heterogeneous in their morphology, growth rates, and potential for metastasis. The possible precursor(s) of the different phenotypes are still unknown. These differences may arise in part as a result of the many different cells from which a given hepatocellular carcinoma may occur. These include first mature (or dividing) hepatocytes, oval cells (periductular cells) (stem cells found adjacent to the ducts of Hering), and potentially stem cells of bone marrow origin present within the liver. Moreover, it is possible that in individuals with multifocal or asynchronous hepatocellular tumors each tumor may have a different cellular origin which can account for their different morphogenesis and biologic characteristics.

Essentially all hepatic cancers arise as a consequence of a chromosomal aberration that can arise during cellular proliferation, when cell damage and death have occurred. The specific disruption involved in any particular case or time can vary depending on the presence of one or more epigenetic or

genetic abnormalities that are present and disrupt the normal regeneration process.

Under normal conditions, the cell cycle is tightly regulated by various phosphorylating enzymes and is promoted by a variety of proteins termed cyclins which when combined with a phosphorylated kinase form a complete catalytic complex that controls cellular regeneration at various points in the cell cycle. Other proteins regulate programmed cell death (apoptosis) which limits cellular regeneration and proliferation.

Inflammation induces cellular injury on one hand and cytokine production and secretion that can result in an enhancement of cellular regeneration on the other hand. Moreover, normal control mechanisms that regulate the cell cycle *(9)* may be disturbed by repeated inflammatory flairs. Regardless of the specific etiology, hepatocellular carcinoma only develops when the control mechanisms regulating cell cycling and renewal or death are disrupted. These disruptions are multiple and include both epigenetic and genetic effects. The various epigenetic effects that can lead to an increased transcription of an oncogene or its promotion are either an increased transcription or a reduced degradation of a cyclin, DNA, RNA, or regulatory protein as a consequence of either hyper- or hypomethylation of DNA or RNA and free-radical injury (peroxidation) as a consequence of a reactive oxygen (ROS) or nitrosyl (RNS) species that occurs as a consequence of oxidative stress. Ultimately, epigenetic processes lead to genetic defects that result in cell cycle disruption.

The principal mechanism by which a nonviral metabolic liver disease progresses to cirrhosis and ultimately hepatocellular carcinoma is a result of oxidative stress induced as a result of cell injury, inflammation, followed by disturbed cellular regeneration and proliferation or reduced apoptosis.

2. OXIDATIVE STRESS

Reactive oxygen species (ROS) and reactive nitrosyl species (RNS) are unstable short-lived molecules generated by oxygen-utilizing cells. They are produced in either the mitochondria or the endoplasmic reticulum as a consequence of stress along an oxygen-utilizing metabolic pathway which contains an electron transport chain or as a result of metabolism involving either a cytochrome P450 enzyme system, xanthine oxidase, nitrous oxide synthesis, lipoxygenase, cyclooxygenase, or NADPH oxidase. Mitochondria because of their role in energy (ATP) production are a major source of ROS which are generated at two sites within the electron transfer (respiratory) chain within mitochondria: complex I (NADH/ubiquinone oxidoreductase) and complex III (ubiquinone/cytochrome oxidoreductase). Of these two sites, the more important is complex I where molecular oxygen (O2) is

converted to singlet oxygen (O^-) by the mitochondrial P450 cytochrome system in the liver, kidney, and to a lesser degree muscle resulting in the generation of ROS when stressed by either an excessive metabolic load (substrate requiring oxidation by mitochondria) or as a result of a reduced antioxidant (particularly glutathione) supply within mitochondria. When glutathione levels are inadequate, the catabolism of hydrogen peroxide (H_2O_2) within mitochondria is reduced as mitochondria do not contain catalase, the enzyme principally responsible for metabolizing H_2O_2. As a result, the unmetabolized H_2O_2 reacts with ferrous (Fe^{+2}) to produce the highly toxic hydroxyl (OH^-) radical. Singlet oxygen (O^-) can react with ROS and RNS activating cell-signaling pathways associated with kinase-linked receptors resulting in phosphorylation of growth-regulating pathways. They also oxidatively alter proteins, DNA, RNA, and lipids which can alter enzyme activity, alter both transcription and translation mechanisms, induce DNA strand breaks, and alter lipid structure and function. Each of these mechanisms disrupts normal cellular function. Moreover, each of these disruptions of critical cellular molecular mechanisms occurs not just in isolation in one cell but rather all together under conditions of oxidative stress amplifying the resultant cellular disruption that occurs.

The transition metals (iron and copper) which are abundant in liver cells accelerate the generation of ROS and RNS and activate the conversion of lipid peroxides into alkoxyl- and peroxyl-radicals which are highly reactive and have a longer half-life than the primary ROS and RNS. These same metals accumulate excessively in many liver disease conditions (hemochromatosis, Wilson's disease, alcoholic liver disease, nonalcoholic fatty liver disease, and nonalcoholic steatonecrosis, and any disease process associated with chronic cholestasis) and can contribute, at least in part, to the summation of events leading to the development of hepatocellular carcinoma in individuals with a metabolic liver disease as it happens when continuous toxic exposure takes place such as under continuous aflatoxin intake with the food.

3. ALCOHOLIC LIVER DISEASE AND HEPATOCELLULAR CARCINOMA

Alcoholic liver disease is composed of a spectrum of histological pathologies ranging from macrovesicular steatosis (fatty liver) to alcoholic hepatitis (fat, inflammation with polymorphonuclear leukocytes, a characteristic sinusoidal fibrosis, and the presence of Mallory bodies in ballooned hepatocytes) to alcoholic hepatitis plus cirrhosis and hepatocellular carcinoma occurring in cases with cirrhosis with or without alcoholic hepatitis *(10)*. Individuals with each of these histopathologic conditions can be either asymptomatic

or symptomatic. In general, the liver injury tests in alcoholic liver disease are characterized by an AST level greater than that of the ALT value. The alkaline phosphatase levels are highly variable depending on the severity of injury, presence of cirrhosis, and presence of bile duct injury/destruction. Hepatic cancer develops most frequently in those with cirrhosis with or without associated alcoholic hepatitis. Approximately 10–15% of alcoholics develop cirrhosis and HCC occurs in 15–20% of these cases at a rate of 3–4% per year. However, HCC can also develop in individuals consuming daily amounts of alcohol without apparent development of cirrhosis.

The role of chronic hepatitis C, and to a much lesser degree the presence of chronic hepatitis B (either evident or occult) in the pathogenesis of primary hepatic cancer in individuals with alcoholic liver disease, remains unclear but may well account for many of the cases of hepatocellular carcinoma in this population. This, however, does not negate the role of alcohol per se in initiating various metabolic changes that contribute to the pathogenesis of hepatic cancer in individuals with alcoholic liver disease. The pathogenetic mechanisms responsible for the development of primary hepatic cancer in cases of HBV and HCV are presented in other chapters and the reader is referred to those chapters for details. These mechanisms are likely to be additive and potentially synergistic to those due to alcohol abuse occurring in cases with alcoholic cirrhosis alone.

As a consequence of ethanol and acetaldehyde oxidation, an oxidative stress is induced in the liver which, if excessive and/or continuous as is the case in alcoholic individuals, results in mitochondrial and endoplasmic reticular injury, resulting in reduced ATP production and cell as well as organelle membrane disruption. These cellular and organelle changes occur in part as a consequence of membrane phospholipids and protein oxidation manifested as lipid peroxidation, protein carbonyl formation, the production of 1-hydroxyethanol radical, and other alkyl-free radicals *(11, 12)*.

Alcohol is not a carcinogen per se but acts as carcinogenic promoter as a consequence of the oxidative stress it induces and the downstream effects of the oxidative stress on cellular lipids, proteins, signaling pathways, DNA and RNA, and subsequent transcription and translation mechanisms. Alcohol-induced reductions in tissue folate levels enhance these effects by impairing transmethylation pathways *(13)*. A reduction in the level of cellular pyridoxaldol 5-phosphate induced by alcohol abuse is also important *(14, 15)*. Each of these effects results in enhanced DNA hypomethylation and upregulated gene expression particularly of proto-oncogenes and subsequently activated oncogenes *(16–19)*.

DNA methylation occurs predominantly at the fifth carbon atom of cytosine–guanine pairings *(20)*. This dinucleotide pairing frequently occurs within the promoter region of genes. Hypermethylation silences gene expression while hypomethylation which can occurs as a result of alcohol

abuse and its effect on folate, pyridoxine, and methionine metabolism is enhanced or unregulated gene expression. This enhanced gene expression and/or enhanced promoter activity enables enhanced binding of transcription factors to DNA and ultimately increased gene transcription *(21, 22)*.

Methionine adenosyltransferase (MAT) is the enzyme responsible for the synthesis of *S*-adenosyl methionine (SAMe). SAMe is the principal biological methyl donor and a precursor of aminopropyl groups utilized in polyamine synthesis and eventually DNA and RNA *(23)*. As such, it is an active participant in biochemical reactions essential for normal cellular proliferation. SAMe is also a precursor of glutathione, a major tissue antioxidant. MAT exists in two isoforms—MAT-1 and MAT-2 *(24, 25)*. MAT-1 is expressed primarily in the liver of adults while MAT-2 is expressed predominately in fetal liver. MAT-2 expression is enhanced in alcoholic liver disease and in human hepatoma and is associated with a reduction in MAT-1 *(26, 27)*. This enhanced MAT-2 expression is due to hypomethylation of the cytosine–guanine dinucleotide pair present in the MAT-2 promoter. This same promoter region has binding sites for heat-shock transcription factor, a STAT (signal transducer and activator of transcription), c-Myb, v-Myb, and GATA consensus binding sites, all of which enhance MAT-2 expression and upregulation of cellular proliferation *(28, 29)*. As a result of the different kinetic characteristics of MAT-1 and MAT-2, liver cells rich in MAT-2, have an overall greater MAT activity at physiologic concentrations of methionine and enhanced proliferative activity, critical factors in the progression from a dysplastic to a neoplastic cell and ultimately the pathway to hepatocarcinogenesis *(30, 31)*.

Each of these consequences of alcohol abuse (folate and B_6 deficiency, oxidative stress, MAT-2 induction, and many as-yet unrecognized adverse cellular events of alcohol abuse occurring in a cirrhotic) contributes to the pathogenesis of hepatocellular carcinoma in the alcoholic cirrhotic. As in the case of the synergism between alcohol and viruses, synergism can take place between alcohol and other substances contained in alcoholic beverages or alcohol and toxins like aflatoxin.

4. NONALCOHOLIC FATTY LIVER DISEASE (NAFLD), NONALCOHOLIC STEATONECROSIS (NASH), AND HEPTOCELLULAR CARCINOMA (HCC)

NASH was described by Ludwig and associates in 1980 *(32)*. In this initial report, the presence of obesity and type II diabetes mellitus as frequent comorbid conditions was recognized. Subsequently, the entire spectrum of NAFLD was recognized to include simple fatty liver, NASH, cirrhosis, and HCC. NAFLD per se is believed to be an innocuous health problem without sequelae, albeit an important and possibly the earliest clinical manifestation

of the metabolic syndrome. As a result of the increasing prevalence of obesity over the last two decades, NAFLD has become recognized as the most frequently recognized clinical hepatic disease in the western world being present in up to 20% of the adult population *(33)*. NAFLD can progress to NASH which is not an innocuous process but has the potential to progress to cirrhosis with NASH or cryptogenic cirrhosis, both of which can develop HCC without any residual histologic evidence of NAFLD. NASH is reported to be present in 3% of the adult population in the United States, a rate twice that of chronic hepatitis C (HCV) *(34)*. As a result, NAFLD and NASH are the two most common hepatic diseases occurring in adults in the United States and Western Europe. Most disturbing is the increased recognition of both NAFLD and NASH in children and adolescents *(33–36)*. Whether this increase in NAFLD and NASH in children will lead to an earlier age of onset of hepatoma in the adult population in the future remains to be determined. The development of NASH in adults is clearly associated with an increased risk of hepatocellular cancer *(37)*.

The metabolic syndrome is characterized by the presence of three or more of the following disease components: NAFLD, type II diabetes mellitus, hypertension, hyperlipidemia, especially hypertriglyceridemia, obesity, coronary artery disease, hyperuricacidemia, sleep apnea, and polycystic ovarian disease *(38)*. Typically, more than three of these disease processes exist in an individual with the metabolic syndrome. Obesity occurs in 30–100% of cases; type II diabetes mellitus occurs in 10–75% of cases; and hyperlipidemia in 2–50% in both adults and children with the syndrome. Coronary artery disease, hypertension, hyperuricacidemia, and polycystic ovarian disease can occur in children and adolescents with NAFLD/NASH but do so considerably less frequently than in adults. It should be noted that NAFLD and NASH can occur in lean individuals, with 3% of documented cases occurring in this population *(29)*. The obesity in individuals with the metabolic syndrome and NAFLD and/or NASH is typically truncal in character.

The recognition of the association between NASH and HCC appears to account in large measure for the observed increase in HCC rates in the United States particularly if cases with HCV disease and HCC are excluded from the calculation. Not only is NASH independently associated with HCC but it appears to enhance the risk of HCC development in cases of HCV-associated cirrhosis *(40)*. The rate at which HCC develops in NASH is not known but can be expected to parallel that seen in alcoholic liver diseases.

The pathophysiologic mechanisms that account for the development of NAFLD and its progression to NASH as well as the downstream complications of cirrhosis and HCC are not entirely clear but appear to be a consequence of a putative two hit processes *(41)*. The first hit is most likely an increase in hepatic fat as a consequence of hypertriglyceridemia. The oppo-

site may be the result of insulin resistance. Insulin resistance is known to lead to a diffuse reduction in tyrosine phosphorylation *(42, 23)* and a resultant disruption in cellular pathways affecting cell growth and differentiation. Triglycerides and fatty acids in the liver induce lipid peroxidation mechanisms as a result of an induction of P450 2E1 and 4A; a disruption of mitochondrial production of ATP; the induction, production, and secretion of inflammatory cytokines (IL-6, IL-8, TNF alpha); and enhanced lipopolysaccharide (LPS) hepato-toxicity *(44–47)*. Each of these events contributes to a state of considerable oxidative stress. As a result of the combination of lipid peroxidation, the production of reactive oxygen species (ROS), and reactive nitrosyl species (RNS), a reduction in hepatic and particularly mitochondrial antioxidants especially glutathione and ultimately a loss of mitochondrial energy production manifested by a loss of ATP production occurs. The latter event dramatically impairs endogenous attempts at cellular injury repair mechanisms. As a net result of this oxidative stress, both genetic and epigenetic mechanisms that contribute to carcinogenesis become manifest.

Importantly the risk of HCC in NASH-affected individuals appears to be limited to those with cirrhosis with or without concurrent NASH. As a result, screening for HCC is indicated only in those with cirrhosis. In such cases, the additional clinical findings of portal hypertension complicated by splenomegaly and thrombocytopenia (<75,000/μl) mandates surveillance for hepatic cancer and should be repeated at 6–12-month intervals utilizing hepatic ultrasound or triple-phase CT scanning procedures. In cases with either an iodine or an intravenous contrast allergy, an annual MRI with an iron-containing contrast agent can be substituted for the triple-phase CT scan.

5. HEMOCHROMATOSIS AND WILSON'S DISEASE AND HCC

Both iron and copper have the potential to be mutagenic as a result of oxidative stress *(48)*. An abundance of DNA adducts has been identified in the hepatic tissue of individuals with hemochromatosis and Wilson's disease *(49)*. DNA damage of hepatocytes exposed to iron has been demonstrated in vitro and most probably also occurs with copper exposure. Classic hemochromatosis is a common autosomal recessive disorder that occurs at a rate of 1/1,000 and is associated with the presence of abnormal alleles for HFE expression. These are C282Y, H63D, and S65C. The latter two alleles are very weakly associated with clinical iron storage and hepatic disease. The synergistic effects between increased hepatic iron storage and other toxins (e.g., alcohol) cannot be excluded as initiators of the hepatic cancerogenic process.

Other causes of "hemochromatosis" include juvenile hemochromatosis (a defect in hemojuvelin), transferrin receptor deficiency, and congenital atransferrinemia. Wilson's disease is also an autosomal recessive disorder that occurs at a rate of 1/30,000. It is due to a defective gene for a P-type ATPase. More than 100 different mutations for this disorder have been identified. The disease can present as an acute hemolytic process with fulminate liver failure, chronic hepatitis, cirrhosis with portal hypertension, or as a psychiatric/ neurologic disorder.

As noted in an earlier section of this chapter, mitochondrial production of ROS and RNS occurring as a consequence of oxidative stress represents a prime source of reactive species in the liver of individuals with either hemochromatosis or Wilson's disease. In both diseases, biochemical (functional) and histological disruption of mitochondria can be demonstrated and contribute to an increased rate of apoptosis, enhanced cellular replication, and a disruption in normal cell cycle functioning.

In Europeans with hemochromatosis, an increased frequency of the p53 tumor suppressor mutation has been reported and contributes to reduced hepatic DNA repair, further enhancing the development for a hepatic cancer *(50, 51)*.

Hepatocellular carcinoma is reported in 7.5–30% of cases of hemochromatosis *(32, 57, 58, 59, 60)*. Almost all the cases have been reported in cirrhotics but at least two cases have been reported in noncirrhotics (32 J). Age >55, the presence of concomitant diabetes mellitus, HbsAg, and alcohol abuse each increases the risk of cirrhosis and hepatocellular carcinoma in individuals with hemochromatosis. Iron reduction therapy was not been associated with a reduced risk of hepatocellular carcinoma in cirrhotics. Hepatocellular carcinoma was found to occur in cirrhotic livers denied of iron at the time of autopsy. Effective iron reduction therapy prevents cirrhosis and therefore also reduces the risk of HCC in individuals with hemochromatosis and most certainly contributes to the lower risk of HCC reported in more recent large cohorts of individuals with hemochromatosis *(32, 57, 58)*.

The development of diabetes mellitus in individuals with hemochromatosis and the observation of macrovascular fat and hyperglycogenation in individuals with Wilson's disease suggest that many, if not all, of the mechanisms that contribute to HCC in individuals with NASH may also be contributory mechanisms to the development of hepatocarcinogenesis in both hemochromatosis and Wilson's disease *(52–61)*.

6. AFLATOXIN-ASSOCIATED HCC

Aflatoxin ingestion is high in areas of Southeast Asia and sub-Saharan Africa where grains and rice are a primary food source. The same is the case in China. These same areas typically store grains for prolonged periods and

as a result the grain often becomes contaminated with fungi that produce aflatoxins. These same geographic regions have high rates of HCC wherein a specific p53 mutation (6^{24gt}) is found *(62)*.

Aflatoxin is metabolized to a potential mutagenic intermediate, aflatoxin 8, 9-epoxide, which is normally detoxified by microsomal peroxide hydrolyses and glutathione *S*-transferase *(62)*. Failure to detoxify this mutagenic intermediate has been known to be associated with the identical p53 mutation found in individuals with HCC within these same geographic areas. Moreover, individuals in these geographic regions have an increased rate of inherited isoforms of both microsomal peroxide hydrolyses and glutathione *S*-transferase with either reduced or no activity of these two enzymes *(62)*. Finally, it needs to be pointed out that these same geographic areas have very high rates of HBV infection. Thus, an interaction between the mechanisms leading to hepatocarcinogenesis in individuals with HBV infection described elsewhere in this textbook and those reported for p53 inactivation by aflatoxin and its metabolite may contribute to the increased development of HCC in these regions of the world.

7. ALPHA 1 ANTITRYPSIN DEFICIENCY AND HCC

Alpha 1 antitrypsin deficiency is an autosomal recessive disorder resulting from a single gene defect wherein a defective gene, with either a Z, S, F, or null allele, occurs in either a homozygous or a compound heterozygous state resulting in reduced plasma serine protease activity. As a result, circulating levels of the serine protease, alpha 1 antitrypsin protein, are reduced to 15–60% of normal *(63)* and the protein accumulates in the endoplasmic reticulum of the liver *(64, 65)*. In addition, mitochondria dysfunction and autophagy occur and contribute to the overall hepatic dysfunction and resultant disease progression *(66)*. The underlying pathophysiology is that of an abnormal folding of the protein and its subsequent accumulation in the endoplasmic reticulum that induces an oxidative stress within both the endoplasmic reticulum and mitochondria. The oxidative stress reaction appears to be a consequence of activation of NF-kB, endoplasmic reticular caspase B cell receptor-associated protein 31, and organelle autophagy.

Most clinical cases of alpha 1 antitrypsin deficiency occur in childhood and are manifested as either a transient acute liver failure or a progressive hepatitis resulting in cirrhosis. It is also seen in adults with late onset of portal hypertension and hepatic synthetic dysfunction *(62–72)*.

Hepatocellular carcinoma is common in adults with alpha 1 antitrypsin deficiency after age 50 where it occurs in 31–67% of all cases having cirrhosis with evidence of overt portal hypertension.

More prevalent than homo zygous alpha 1 antitrypsin deficiency is the occurrence of the heterozygous state with either a single Z, S, or F allele and a normal allele. This situation is not directly associated with liver disease

but appears to act as a potentiating factor for liver disease and liver disease progression as well as HCC when it occurs in association with any of a number of other liver disease processes such as HBV, HCV, alcohol, and NASH. The combination of these various other hepatocarcinogenic mechanisms in patients with alpha 1 antitrypsin deficiency may act in an additive or synergistic way and lead to the development a hepatic cancer. As is the case with NAFLD, hemochromatosis, and Wilson's disease, HCC only occurs in those cases that are cirrhotic. Thus, screening and surveillance for HCC need not be instituted until clinical evidence of cirrhosis is present.

8. FAMILIAL INTRAHEPATIC CHOLESTASIS

Each of these diseases is a result of an autosomal recessive disorder resulting in defective hepatocyte canalicular membrane transport.

(A) Progressive familial intrahepatic cholestasis type I was originally described by Byler and has been termed Byler's disease as a result *(73)*. It is a mutation in the FIC-1 gene (ATP8B1) and results in a spectrum of liver diseases ranging from a benign condition with intermittent pruritus with or without jaundice termed benign recurrent intrahepatic cholestasis (BRIC) to severe intractable pruritus, jaundice, and liver failure. Genotype/phenotype correlations have documented more severe mutations in individuals manifesting the PFIC-1 phenotype syndrome than those manifesting the BRIC phenotype, which is characterized by more missense mutations *(74)*. With advanced cholestasis HCC can occur in these cases.

(B) Bile salt export protein (BSEP) deficiency is a result of an autosomal recessive disorder in bile salt secretion due to a defective bile salt export protein which is liver specific unlike that occurring in PFIC-1 *(75)*. Specifically, the disease is due to a mutation in an adenosine triphosphate-binding cassette transporter gene (ABCB11), the principal canalicular transporter of bile acids into bile. Disease severity varies inversely as a function of the degree of BSEP expression. In severe cases, the disorder is termed PFIC-2 and in less severe cases it is termed BRIC-2. Cases of HCC have been reported in the severe forms of BSEP deficiency *(76)*.

(C) Multidrug resistance-3 (MDR-3) deficiency or PFIC-3 is a consequence of a mutant class III multidrug resistance p-glycoprotein identified as MDR-3 (ABCB4) which is responsible for canalicular phospholipid transport *(77)*. Its clinical manifestation is highly variable with clinical onset of disease occurring between ages 1 month to 20 or more years. Unlike the proceeding two conditions that have low levels of gamma-glutamyl transpeptidase despite cholestasis, this disorder is character-

ized by an elevated gamma-glutamyl transpeptidase level. Hepatic cancer can occur in this disorder but its frequency is much less than in the other two forms of familial cholestasis.

9. BILE ACID SYNTHEIC DISORDERS AND HEPATOCELLULAR CARCINOMA

Nine distinct genetic disorders of bile acid synthesis have been identified and characterized clinically *(78)*. All are inherited as an autosomal recessive disorder. They occur as a result of either a specific enzyme deficiency that is unique for normal bile acid synthesis or a disruption in peroxisomal function.

Those due to a defect in bile acid synthesis can be treated medically, but if unrecognized or untreated can progress to cirrhosis and liver failure *(79)*. Liver cancer can occur in these cases but is unusual as liver failure leads to an early death in untreated cases, and autopsies which are likely to identify HCC have rarely been performed in these cases.

The hydrophobic bile acids that accumulate as a result of cholestasis of any cause are known to enhance apoptosis by activating caspases and disrupting the balance between cell cycle renewal and apoptosis. Bile acids also enhance mitogen-activated protein kinase (MAPK) activation dependent on epidermal growth factor receptor activation which enhances cellular regeneration/proliferation mechanisms. The net effect of these two different bile acid-induced mechanisms in individuals with metabolic disease, particularly those metabolic disease with cholestasis, positively affects cell cycle regulation, enhancing cell proliferation and the opportunity for the development of a hepatocellular carcinoma. Both macrophages and neutrophils present in inflammatory tissue can produce ROS and have a cytosolic myeloperoxidase that produces hydrochloride, a powerful oxidant. These cells accumulate within the liver of individuals with various hepatic diseases including essentially every metabolic liver disease and contribute to the overall oxidative stress experienced by the liver.

No therapy exists for those with defective peroxisomal dysfunction. The liver disease in this subset of cases is only a part of the overall disease process wherein the clinical manifestations occur and involve the nervous system and the adrenal glands, as well as the liver.

10. DEFECTS IN CARBOHYDRATE METABOLISM

10.1. Galactosemia

This disorder is characterized by a deficiency of galactose-1-phosphate uridyl transferase. Several different alleles for this disorder have been identified but most cases are due to a single common mutation (Q188R) *(80)*.

The enzymatic defect blocks the metabolism of galactose-1-phosphate and causes hemolysis, jaundice, liver disease, lactic acidosis, renal tubular acidosis, failure to thrive, hepatosplenomegaly, cataracts, and e.coli sepsis particularly in neonates. A single report of HCC in a child with this disorder, who had a transplant, has been reported having not been treated medically, if the child had been treated appropriately with a galactose-free diet clinical liver disease should not have occurred and the hepatic cancer and requirement for a liver transplanted would have been avoided occur *(81)*.

10.2. Hepatic Glycogen Storage Disease

Five different hepatic glycogen storage disorders have been characterized and specifically identified. These are glycogen storage diseases type I, III, IV, VI, and IX. The latter two tend to be mild while the first three, types I, III, and IV, are progressive and can be severe leading to a requirement for liver transplantation *(82)*. Hepatic adenomas and cancer have been reported in types I, III, and IV *(82–86)*. Tumor detection in each disorder is dependent upon imaging procedures.

(i) Glycogen Storage Disease I (GSD-I) is an autosomal recessive disorder with a prevalence of 1/20,000–1/225,000. Glucose 6-phosphate deficiency characterizes GSD-I. The enzyme is expressed on the inner surface of the endoplasmic reticulum. Two distinct enzymatic defects account for this disease. A deficiency of the catalytic compound of the enzyme produces GSD-Ia while a deficiency of the transporter component is responsible for GSD-Ib. The metabolic consequences of the two are identical with the exception that neutropenia occurs with GSD-Ib. Molecular genetic studies are used currently to make the diagnosis and have replaced the older enzymatic activity assays. It is important to note that the latter method of diagnosis can result in a misdiagnosis (failure to identify) of GSD-1B as a result of using frozen tissue that enables the catalytic activity of the endoplasmic reticulum to be assayed and detected but not the transporter component resulting in a false normal result.

Chronic liver disease does not occur in cases of GSD-I but poor metabolic control can result in the development of hepatic adenomas that occasionally degenerate into HCC.

Liver transplantation has been used to treat GSD-I with poor metabolic control with medical measures or as a result of the development of either a hepatic adenoma or a HCC *(51)*.

(ii) Glycogen Storage Disease-III (GSD-III)

Defective glycogen debrancher enzyme characterizes GSD-III. It tends to be milder than type I but also involves muscle and in adults can be manifested with either a severe skeletal myopathy or a cardiomyopathy.

It is an autosomal recessive disorder with a prevalence of 1/20,000–25,000. As was the case with GSD-I, two forms of GSD-III occur. GSD type A involves muscle and liver and represents 85% of the cases. GSD type B accounts for only 15% of cases and involves only the liver.

Cirrhosis can develop in GSD-III unlike type I and liver tumors have been reported in cases with advanced fibrotic liver disease.

(iii) Glycogen Storage Disease IV (GSD-IV)

GSD-IV is an autosomal recessive disorder caused by a deficiency of the glycogen branching enzyme occurring at a rate of 1/20,000–25,000 and results in the accumulation of unbranched glycogen in the liver, heart, muscle, skin, intestines, and nervous systems (both central and peripheral). It typically presents as infantile cirrhosis. HCC has been reported in these cases *(84–86)*.

11. TYROSINEMIA TYPE I

Tyrosinemia type I or hepatorenal tyrosinemia is an autosomal recessive disorder due to a defect in fumarylacetoacetate hydrolase which results in an accumulation of fumarylacetoacetate and maleylacetoacetate *(87)*. It has a prevalence of 1/100,000 worldwide but occurs in specific geographic regions at an increased rate approximately of 1/2000. It presents as acute hepatitis, acute liver failure, or cirrhosis often with a HCC. Apoptosis of hepatocytes is a characteristic feature of the disease *(88)*. The apoptotic signal in tyrosinemia type I appears to be fumarylacetoacetate *(88)*. Both fumarylacetoacetate and malylacetoacetate are alkylating agents that can cause DNA damage. Thus the development of HCC in cases of tyrosinemia type I is due to a combination of DNA and RNA mutagenesis occurring as a consequence of oxidative stress and nucleic acid alkylation *(89–95)*. The oxidative stress is a result of the consumption of antioxidants by malylacetone, fumarylacetone, and succinylacetic acid and succinyl acetone.

The introduction of 2-(2-nitro-4-trichloromethylbenzol)-1,3-cyclohexendrome (NTBC) which blocks tyrosine degradation at 4-hydroxyphenylpyruvate prevents the formation of the alkylating agents fumarylacetoacetate and malylacetoacetate and has greatly altered the natural history of the disease *(96)*. Unfortunately some 10% of cases of tyrosinemia type I do not respond to NTBC therapy and require liver transplantation prior to age 2 if HCC is to be prevented.

12. THE PORPHYRIAS

(A) Acute intermittent porphyria (AIP) is an autosomal dominant disorder resulting from a half normal level of porphobilinogen deaminase. It

is characterized by increased plasma and urinary levels of delta amino levulinic acid and porphobilinogen as well as clinical episodes of recurrent visceral, autonomic, and central neuropathy with abdominal pain. It occurs at a rate of 1/20,000 and is the most common form of porphyria.

(B) Congenital intrahepatic porphyria (CIP) is a very rare autosomal recessive disorder characterized by markedly reduced uroporphyrinogen III synthetase. It has a highly variable age at the time of clinical onset and is characterized by red brown teeth, frequent bacterial infections, and a deposition of iron in the liver and spleen.

(C) Porphyria cutanea tarda (PCT) is an autosomal dominant disorder characterized by reduced levels of uroporphyrinogen decarboxylase. Three different types of the disease are recognized. These are

(1) sporadic (worldwide) occurring at a rate of 1/25,000 in the United States wherein the liver alone is enzyme deficient
(2) familial (autosomal dominant) form that involves enzyme deficiency in the liver and bone marrow
(3) familial (rare autosomal recessive) form that occurs in the liver characterized by sun exposure-induced blistering, dermal scarring, hypo- and hyper pigmentation, hirsutism, and an accumulation of porphyrins in the liver, plasma, and urine. Uroporphyrinogen decarboxylase enzyme activity in the liver can be reduced by iron-dependent oxidative stress induced by alcohol, HCV infection, HIV infection, smoking, and a HFE gene mutation.

(D) Hepatoerythropoietic porphyria (HEP) type II porphyria cutanea tarda is due to a markedly reduced uroporphyrinogen decarboxylase expressed in liver and RBC.

(E) Hereditary coproporphyria (HCP) is autosomal dominant due to reduced activity of coproporphyrinogen oxidase and is characterized by signs and symptoms similar to acute intermittent porphyria but with sun sensitivity manifested by increased urinary coproporphyrins.

(F) Variegate porphyria (VP) in an autosomal dominant disorder characterized by hepatic deficiency of protoporphyrinogen oxidase (PPO). Characterized by neurologic and cutaneous signs and symptoms similar to AIP, it is associated with episodes of severe hyponatremia during attacks.

(H) (G) Erythropoietic protoporphyria (EPP) is an autosomal dominant disorder of ferrochetalase deficiency. It is the third most common form of porphyria. Skin changes are universal with this condition consisting of dermal lichenification and blistering. Protoporphyrins accumulate in the liver and induce a form of biliary cirrhosis.

HCC has been reported to occur in AIP, CIP, PCT, VP, HEP, but not in EPP *(97–105)*.

13. CYSTIC FIBROSIS

Cystic fibrosis is an autosomal recessive disorder that results in the development of abnormal chloride channels and an inability to secrete thin watery secretions in the tracheobronchial tree, intestine, and biliary system. It occurs almost exclusively in Caucasians at a rate of 1/2000–3000 live births. The hepatic manifestations of cystic fibrosis are focal biliary cirrhosis that can become panlobular. The hepatic disease is characterized by cholestasis and inflammation often complicated by episodes of recurrent biliary sepsis. With progressive disease, toxic bile acids accumulate and induce epigenetic alterations that result in defective cell cycle regulation and in rare cases, hepatic cancer in a liver with advanced biliary cirrhosis *(106)*.

The hydrophobic bile acids that accumulate as a result of cholestasis of any cause are known to enhance apoptosis by activating caspases and disrupt the balance between cell cycle situation and apoptosis. Bile acids also enhance mitogen-activated protein kinase (MAPK) activation dependent on epidermal growth factor receptor activation enhancing cellular regeneration/proliferation mechanisms. One net effect of these two bile acid mechanisms in individuals with metabolic diseases particularly those metabolic diseases with cholestasis can affect cell cycle regulation enhancing the opportunity for the development of a hepatocellular carcinoma. Both macrophages and neutrophils can produce ROS and a myeloperoxidase that produces hypochlorite, a powerful oxidant. One or both of these cells accumulate within the liver of individuals with various hepatic diseases including metabolic liver diseases and contribute also to the next oxidant stress experienced by a liver with a metabolic disease.

14. ALAGILLE'S SYNDROME

Alagille's syndrome is an autosomal recessive disorder due to a defect in JAG-1 that results in a paucity of bile ducts and a biliary cirrhosis that can lead to the development of HCC *(107)*. It is characterized by a triangular face, embrotoxin abnormality of the eye, butterfly vertebrae, peripheral pulmonary artery stenosis, and resultant pulmonary hypertension as well as chronic cholestasis.

15. LINKED SIDEROBLASTIC ANEMIA

This disease occurs as a result of a deficient activity of Δ5-aminolevulinic synthetase in the mitochondria of erythroid cells.

As a result ineffective erythropoiesis iron accumulation occurs in the mitochondria of the erythroid cells of the marrow, liver, heart, and joints.

The clinical manifestations of the disease include hepatomegaly, cirrhosis, and HCC, diabetes, hypogonadism, and skin changes similar to hereditary hemochromatosis *(108, 109)*.

16. FANCONI ANEMIA

This is an autosomal recessive disorder characterized by diffuse congenital anomalies, bone marrow failure, and malignancy *(110–113)*. The carrier frequency is 0.5%. Affected individuals are highly sensitive to cross-linking agents and develop numerous chromosomal breaks. The most frequent extra hematologic abnormalities are radial ray defects affecting the distal radius, thumb, hip, vertebrae and knee abnormalities, insulin resistance, and short stature. Liver tumors are common. The roles of androgen therapy, insulin resistance, and DNA repair dysfunction coupled with reduced apoptosis presumably account for the hepatic pathology in this disorder.

17. TYPE II DIABETES MELLITUS

This is a common disorder accounting for >85% of all cases of diabetes mellitus and is typically seen in adults but it also occurs frequently in children especially those manifesting various components of this metabolic syndrome (obesity, hypertension, dyslipidemia, sleep apnea, polycystic ovaries, and gout).

Excessive insulin results in increased growth factor receptor binding protein 2, RAS, RAF, MEK, MAK activation as well as PDK-1 and p70-56 K activation, all of which increase cell proliferation.

These events occurring in conjunction with the adverse effects of hepatic steatosis and the oxidative stress associated with hypertriglyceridemia and free fatty acid increases in the liver and plasma probably account for the mutagenesis which results in the development of hepatocellular carcinoma in cases of type II diabetes mellitus *(114–118)*.

18. HEREDITARY FRUCTOSE INTOLERANCE

Individuals with hereditary fructose tolerance, who survive the neonatal period, can, with repeated fructose challenges, develop fibrosis liver disease and rarely a hepatocellular carcinoma *(119)*.

19. HEREDITARY HEMORRHAGIC TELEANGIECTASIA

This disorder is characterized by vascular lesions in the skin, intestine, and solid organs to include the liver, spleen, kidney, heart, and brain. Typically the disorder presents as recurrent epistaxis. Cardiac failure can occur

with large solid organ artero-venous fistulae. After epistaxis, the major problem is recurrent bleeding necessitating iron and other transfusion therapy. As a result of years of transfusion the development of a blood-borne infection is likely and can result in liver disease and HCC. A rare hepatoma has been reported in patients with this disorder in the absence of a history of hepatitis *(120)*.

20. ADENOSINE DEAMINASE DEFICIENCY

The disorder is a very rare autosomal recessive disorder that results in a severe combined immunodeficiency in children and adolescents. A delayed adult form has been recognized recently and is associated with autoimmune disorders and hepatic dysfunction as well as hepatoma *(121–128)*.

21. STEROID-INDUCED HCC

Estrogens and androgens have both been reported to induce adenomas and hepatomas in the liver. Estrogens are used for the purpose of oral contraception and typically produce adenoma and rarely HCC *(129–131)*.

Androgens are used for their anabolic activity and more often than estrogens produce HCC *(131–135)*.

22. SUMMARY

This chapter discusses the most widely recognized metabolic disorders that are associated with hepatic carcinogenesis. The authors make no assertion that it is all inclusive, rather it presents those that are reasonably well characterized. Other disorders may have random hepatic cancers or liver disease-associated cancers that have yet to be recognized as a frequent occurrence in the disorder as a result of rarity of the metabolic disorder and the low rate of HCC that can occur in them. Thus the recognition of a linkage between the two is very difficult to recognize and quantify.

In all of the disorders recognized and presented herein, the basic metabolic defect includes either a state of oxidative stress or an alteration in cell proliferation or cell death as a downstream consequence of the metabolic defect.

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10 Clinical Features and a Clinician's Diagnostic Approach to Hepatocellular Carcinoma

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CONTENTS

ABSTRACT

The clinical presentation of hepatocellular carcinoma may take on a variety of different forms, ranging from asymptomatic (diagnosed via tumor markers or imaging studies) to a catastrophic hemoperitoneum with shock due to tumor rupture. This chapter will highlight the many diverse clinical manifestations of hepatocellular carcinoma and describe the clinician's role in screening the "at-risk" population. It will touch on the serologic and

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radiologic techniques available and then expound upon the pros and cons of liver biopsy. Finally, primary and secondary chemopreventative strategies will be discussed.

Key Words: Signs and symptoms; paraneoplastic syndromes; screening; liver biopsy; chemoprevention; diagnostic algorithm

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide. It is the tumor with the second highest increase in incidence and with the highest increase in death rates over the last decade in the United States *(1)*. An estimated 1 million new cases are diagnosed every year. Its incidence is increasing because of the long-term consequences of Hepatitis C virus (HCV) and Hepatitis B virus (HBV) infection along with better diagnostic modalities. It has been estimated that the number of cases of HCC will increase by 81% by the year 2020, mostly due to the hepatitis C epidemic in the United States *(2)*.

HCC is frequently diagnosed late in its course because of the absence of pathognomonic symptoms and the liver's large functional reserve *(3)*. Many patients with HCC in high-incidence locations have hepatic decompensation at presentation. Symptomatic HCC has a very poor prognosis, with a median survival of 1–8 months and a 5-year survival rate of only 3% *(4–6)*. Cohort studies have shown that HCC is currently the leading cause of death in patients with cirrhosis *(7)*.

The clinical presentation of HCC is variable from patient to patient. It can range from an asymptomatic presentation to tumor rupture with a catastrophic hemoperitoneum. Hence, screening is very important in the "at-risk" population to prevent HCC. With excellent radiological techniques and supportive serum markers, the diagnostic utility of liver biopsy has come into question. The clinician plays a pivotal role in performing diagnostic testing and in both the primary and the secondary chemoprevention of HCC.

This chapter will discuss the various clinical presentations of HCC, offer an approach to screening and diagnostic testing, debate the pros and cons of histologic evaluation, and finally illustrate primary and secondary chemopreventive strategies that may be employed by the hepatologist.

2. CLINICAL FEATURES

2.1. Asymptomatic

Most cases of HCC appear in the setting of cirrhosis, hence a majority of findings will be similar to those observed in patients with advanced cirrhosis. Due to screening programs for cirrhotic patients, tumors are now

being detected even at an asymptomatic stage. These tumors tend to be smaller (with current imaging modalities, tumors as small as 0.5 cm can be detected) and therefore are more amenable to potentially curative therapies such as resection, transplantation, and tumor ablation *(8)*. The frequency of asymptomatic diagnosis is dependent on the intensity of the screening program. In a series of 461 Italian patients with HCC, 23% were asymptomatic *(9)*.

2.2. Classic Triad

The classical triad for presentation of HCC, though uncommon in clinical practice, includes right upper quadrant abdominal pain, weight loss, and hepatomegaly (see Table 1). Patients with these symptoms usually have a tumor larger than 6 cm on presentation. The pain frequently is described as a dull, continuous ache that intensifies late in the course of the illness. This occurs due to the involvement of the Glisson's capsule. There may be referred pain to the shoulder. Firm, often massive hepatomegaly is a feature of symptomatic malignant liver tumors. On auscultation over the enlarged liver, one may hear an arterial vascular bruit due to increased vascularity in up to 25% of cases. This occurs in systole, is rough in character, and is not affected by changing the position of the patient *(10)*. This finding rarely occurs with hepatic metastases. Less often, a friction rub is heard over the tumor. This sign is more typical of hepatic metastases or abscesses *(11)*.

Table 1
Symptoms and Signs of Hepatocellular Carcinoma

Symptom	*Frequency (%)*	*Sign*	*Frequency (%)*
Abdominal pain	59–95	Hepatomegaly	54–98
Weight loss	34–71	Hepatic bruit	6–25
Weakness	22–53	Ascites	35–61
Abdominal swelling	28–43	Splenomegaly	27–42
Nonspecific gastrointestinal symptoms	25–28	Jaundice	4–35
Jaundice	5–26	Wasting	25–41
		Fever	11–54

Reprinted from Kew *(11)*, p. 1578.

2.3. Hepatic Decompensation

Any patient with known cirrhosis can present with acute hepatic decompensation due to a new HCC. These patients can develop new-onset ascites, variceal hemorrhage (which can be recurrent), progressive encephalopathy, or jaundice. Any of the above features should raise the suspicion for new HCC in the differential diagnosis. Control of ascites may be difficult with standard diuretic therapy and the ascites may often be bloodstained.

2.4. Gastrointestinal Hemorrhage

Approximately 10% of patients with HCC will present with some form of gastrointestinal bleeding at the time of presentation. About 40% of these patients will have esophageal variceal hemorrhage. This occurs due to portal vein thrombosis from direct tumor invasion causing elevated portal pressures. Peptic ulcer disease, portal hypertensive gastropathy, and other benign causes account for the remaining 60% of cases involving bleeding *(12)*. Rarely, the tumor can invade directly into the gastrointestinal tract and cause significant bleeding at presentation.

2.5. Tumor Rupture/Hemoperitoneum

Rarely HCC manifests as an "acute abdomen" when the tumor ruptures, causing a hemoperitoneum. Tumor rupture may occur spontaneously or with minor blunt abdominal trauma. The mechanism of spontaneous HCC rupture has not been fully elucidated. Some investigators believe that disruption of a friable feeding artery or tear in the surface of a tumor under high pressure could cause rupture *(13)*. Another hypothesis is that an increase in intratumoral pressure occurs due to progressive or sudden occlusion of branches of hepatic veins by tumor invasion and that this causes venous congestion within the tumor which in turn may lead to bleeding and rupture.

The clinical presentation is that of severe abdominal pain, vascular collapse, and signs of peritoneal irritation. This type of presentation, although late in the course of the disease, occurs in about 5% of cases. The diagnosis is established by paracentesis, which will reveal bloodstained ascites. CT findings may include the following: hemoperitoneum, HCC with surrounding perihepatic hematoma, active extravasation of contrast material, tumor protrusion from the hepatic surface with focal discontinuity due to rupture through the capsule or the "enucleation sign" with findings of a low-attenuated mass with peripheral rim enhancement *(14)*. The following findings are associated with an increased risk of rupture: a large HCC, a contour

protrusion, and portal vein thrombosis *(14)*. Angiography and embolization of the bleeding vessel by interventional radiology can be a life-saving procedure in these cases *(15)*.

2.6. Paraneoplastic Syndromes

These systemic sequelae result, directly or indirectly, from synthesis and secretion of biologically active substances by the tumor. There is secretion of hormones or hormone-like substances, which cause the clinical effect in these patients. Physical findings of these paraneoplastic conditions should raise clinical suspicion to prevent any delay in diagnosis of HCC as these may precede the local effects of the tumor (see Table 2).

Hypoglycemia (<5% of patients) results from defective processing by malignant hepatocytes of the precursor of insulin-like growth factor II (pro-IGF-II). The resulting big IGF-II circulates in 60-kDa complexes that are appreciably smaller than the normal complexes *(16)*. With easier transfer across capillary membranes, the effect is to increase glucose uptake by the tissues with resultant hypoglycemia. *Polycythemia* (<10% of patients) is caused by synthesis of an erythropoietin-like substance by malignant hepatocytes *(17)*. Patients with HCC, especially the sclerosing variety, may present with *hypercalcemia* in the absence of osteolytic metastases. The

Table 2
Paraneoplastic Syndromes Associated with HCC

Hypoglycemia
Polycythemia (erythrocytosis)
Hypercalcemia
Sexual changes: isosexual precocity, gynecomastia, feminization
Systemic arterial hypertension
Watery diarrhea syndrome
Carcinoid syndrome
Osteoporosis
Hypertrophic osteoarthropathy
Thyrotoxicosis
Hypercholesterolemia
Thrombophlebitis migrans
Polymyositis
Neuropathy
Cutaneous manifestations: pityriasis rotunda, Leser–Trelat sign, dermatomyositis, pemphigus foliaceus, porphyria cutanea tarda

Adapted from Kew *(11)*, p. 1579.

probable cause is secretion of parathyroid hormone-related protein by the tumor *(18)*. *Arterial hypertension* complicating HCC is the result of ectopic synthesis of angiotensinogen by malignant hepatocytes *(19)*. *Feminization* results from the tumor's conversion of circulating dehydroepiandrosterone to estrone and, to a lesser extent, estradiol *(20)*. *Hypercholesterolemia* is the result of autonomous de novo synthesis of cholesterol by the tumor *(21)*. *Watery diarrhea,* which may be severe and intractable, is probably related to secretion of a peptide that promotes intestinal secretion, e.g., vasoactive intestinal peptide (VIP), gastrin, and prostaglandins *(22)*.

Several cutaneous manifestations have been described in association with HCC; however, none is specific for the diagnosis. These include dermatomyositis, pemphigus foliaceus, sign of Leser–Trelat, pityriasis rotunda, and porphyria cutanea tarda *(23)*. *Pityriasis rotunda* may be a useful marker of HCC in black Africans. The rash consists of single or multiple, round or oval, hyperpigmented, scaly lesions on the trunk and thighs that range in diameter from 0.5 to 25 cm *(24)*.

2.7. Other Rare Manifestations

HCC can cause fever of unknown origin *(25)*. Massive tense ascites resulting from hepatic vein spread (Budd–Chiari syndrome) *(26)* and obstructive jaundice resulting from bile duct compression are complications of locally advanced tumor. Bone pain and sudden paraplegia with vertebral destruction can occur due to distant metastasis. Pulmonary metastases can present with cough and dyspnea in patients with advanced HCC.

3. SCREENING FOR HCC

More than 80% of cases of HCC occur in a background of cirrhosis *(27)*. Major causes of cirrhosis are HBV, HCV, and alcohol. Less prevalent conditions such as hemochromatosis, primary biliary cirrhosis, nonalcoholic steatohepatitis, autoimmune hepatitis, and Wilson disease have also been associated with the development of HCC. There are no large prospective studies to determine the incidence of HCC among patients who have cirrhosis from such less prevalent conditions, but their risk is significantly higher than that of the normal population. It is higher in males, patients older than 50 years and with increased α-fetoprotein (AFP) concentration. Smoking slightly increases the oncogenic risk *(28)* whereas coffee consumption seems to reduce the risk *(29)*.

In a randomized controlled trial of nearly 19,000 HBV-infected patients in China, it was shown that HCC surveillance with testing of serum AFP and performance of abdominal ultrasound (US) at repeated 6-month intervals

improves survival *(30)*. In that particular study the adherence to surveillance was relatively low (<60%), a 37% reduction in HCC-related mortality was reported. AFP and liver US are the most widely used and recommended tools for HCC screening at 6 month intervals in high-risk groups. These screening guidelines have been endorsed by both the European Association for the Study of the Liver (EASL) *(7)* and the American Association for the Study of Liver Diseases (AASLD) *(31)*.

"High-risk" patients identified in the AASLD practice guidelines include *(31)*

Hepatitis B carriers (HBSAg positive)

(1) Asian males ≥ 40 years
(2) Asian females ≥ 50 years
(3) All cirrhotic hepatitis B carriers
(4) Family history of HCC
(5) Africans over age 20 years
(6) Severity of underlying liver disease: current and past inflammatory activity, high HBV DNA concentrations remain at risk for HCC

Non-hepatitis B cirrhosis

(1) Hepatitis C
(2) Alcoholic cirrhosis
(3) Genetic hemochromatosis
(4) Primary biliary cirrhosis

Inadequate data preclude a risk assessment in the following groups:

(a) Alpha-1 antitrypsin deficiency
(b) Nonalcoholic steatohepatitis
(c) Autoimmune hepatitis

The cost-effectiveness of HCC surveillance strategies using both AFP and US has been evaluated in retrospective studies as well as mathematical models *(31–33)*. In patients with compensated cirrhosis there might be modest gain in quality-adjusted life years at acceptable costs. In patients undergoing HCC screening while awaiting liver transplantation, screening with computerized tomography is associated with the greatest gain in life expectancy and is possibly cost-effective in this setting *(34)*.

So, current recommendations for HCC screening as proposed by the AASLD are as follows *(31)*:

(1) Surveillance for HCC should be performed using ultrasonography (level II).
(2) AFP alone should not be used for screening unless ultrasound is not available (level II).

(3) Patients should be screened at 6- to 12-month intervals (level II).
(4) The surveillance interval does not need to be shortened for patients at higher risk for HCC (level III).

4. DIAGNOSTIC APPROACH

If there is a clinical suspicion that a patient may have HCC or with an abnormal screening test, imaging becomes a very important next step in the diagnosis, staging, and management of HCC. The most reliable diagnostic tests are either a triple-phase helical CT or a triple-phase dynamic contrast-enhanced magnetic resonance imaging (MRI) *(35*, *36)*. Hepatic angiography has fallen out of favor in recent times.

The most important feature of HCC during CT scan or MRI is the presence of arterial enhancement followed by delayed hypointensity of the tumor in the portal venous phase and delayed phases of imaging. This is also called the "washout phase" of imaging *(37)*. HCC derives its blood supply from the hepatic artery, whereas the remainder of the unaffected liver receives its blood supply from both the hepatic artery and the portal vein. The presence of arterial enhancement followed by washout phase has a sensitivity of 90% and specificity of 95% *(38)*. However, 71% of patients with HCC will have this classical arterial enhancement and washout on one of the test, whereas the rest do not have these features *(38)*. Hence, these patients may require a liver biopsy for the diagnosis of HCC. There have been four studies, using the explanted liver as a gold standard, showing that MRI is slightly better in the characterization and diagnosis of HCC when compared with CT scan *(39–42)*. The diagnostic accuracy is affected by the size of the lesions. For tumors larger than 2 cm in size, MRI is reported to have an accuracy of >90%; however, for tumors smaller than 2 cm, it is reduced to 33% *(43)*.

An AFP serum level above 200 ng/ml is highly specific for HCC diagnosis in patients with cirrhosis and with radiological evidence of hepatic lesions *(31)*. However, AFP lacks sensitivity as it has been reported that only one-third of patients with HCC will have AFP levels higher than 100 ng/ml *(44*, *45)*. Overall, AFP is elevated in approximately 60–70% of patients with HCC in the United States and Europe *(46)*. Because of this, serum AFP falls short of being an ideal tumor marker.

A diagnostic approach to HCC has been developed by expert consensus based on data gleaned from the literature. The evaluation includes serology, cytohistology, and radiologic testing. Some form of imaging, such as CT scan or MRI, is always required to determine the extent of disease. In the correct setting of Hepatitis B or cirrhosis of other etiology, a mass found

incidentally or on screening ultrasound has a high likelihood of being HCC. The sequence of tests used to diagnose HCC depends on the size of the lesion *(31)* (see Fig. 1).

5. ROLE OF LIVER BIOPSY – "TO NEEDLE OR NOT TO NEEDLE"

Patients with compensated cirrhosis and low Model for End Stage Liver Disease (MELD) scores usually would not undergo liver transplantation unless they have HCC. Hence, presence of HCC should be definitely ascertained before deciding on the necessity of liver transplantation in this group of patients *(47)*. Current imaging techniques allow detection of small liver nodules (<1 cm), but not all liver nodules between 1 and 2 cm are HCC. Generally, a definitive diagnosis of HCC can be made without tissue analysis in case of nodules >2 cm when they have a characteristic pattern on either computed tomography or magnetic resonance imaging. However, for lesions between 1 and 2 cm, two concordant imaging techniques are needed. Otherwise, these lesions should not be treated as HCC without histological evidence because of a rate of false positive as high as 20% *(48*, *49)*. Biopsy may be needed for making a diagnosis of HCC in patients with cirrhosis with nodules that do not fulfill the above criteria (see Fig. 1). Lesions <1 cm in size may be especially difficult to characterize, even with the best imaging techniques. Hence these lesions should be followed very closely with imaging in 3–4 months in order to detect growth suggestive of malignancy (see Fig. 1). Lack of growth over a period of more than 1–2 years suggests that the lesion is not HCC.

United Network of Organ Sharing (UNOS) allows an allocation of 22 MELD points for a T2 HCC lesion, said to be within the "Milan criteria" *(50)*. This is defined as a single lesion greater than 2 cm but less than 5 cm or up to three lesions, each less than 3 cm. There also cannot be evidence of portal vein invasion or metastatic tumor spread in order to qualify for this MELD upgrade. For every 3 months on the transplant list, UNOS allows the patient an additional 3 points if the lesion remains at a T2 stage on follow-up imaging studies.

The transplant community has commonly believed that US-guided biopsy should be avoided in candidates for transplantation with suspected HCC. This is because of the risk of needle tract seeding and also a risk of hematogenous spread during liver biopsy *(47)*. Post-transplantation immunosuppression accelerates tumor growth and increases the risk of tumor recurrence in cases of dissemination. Hence, the risk must be balanced against the risk of futile liver transplantation in patients who do not have a malignancy *(47)*.

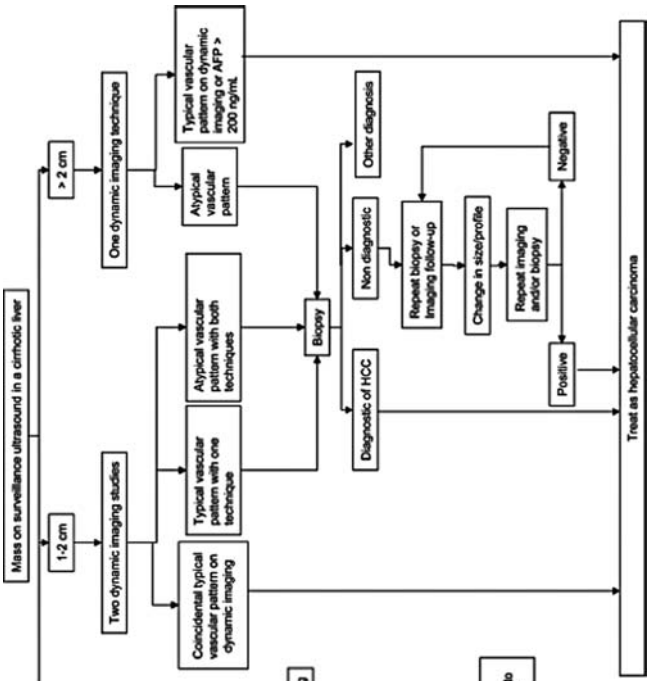

Fig. 1. The figure illustrates an algorithm for investigation of a nodule found on ultrasound during screening or surveillance as endorsed by the AASLD practice guidelines *(31)*. Reprinted from Bruix J and Sherman M. AASLD Practice Guideline: Management of Hepatocellular Carcinoma. *Hepatology* 2005; 42(5): 1208–1236 *(31)*.

5.1. Importance of Liver Biopsy

Several biopsy procedures have been developed to obtain tissue sample, including image-guided fine-needle aspiration (FNA) biopsy, blind or guided percutaneous needle core biopsy, and transjugular needle core biopsy.

The overall sensitivity and accuracy of US-guided biopsy generally exceeds 85% *(47)*. There are virtually no false-positive findings. The negative predictive value of biopsy remains low. Therefore, in patients with negative biopsy findings, HCC cannot be definitely ruled out. These patients should undergo enhanced surveillance or a second liver biopsy. However, one study has suggested that a second liver biopsy performed immediately after the first one has a limited chance of success, with a gain of about 35% *(51)*. Rate of false negatives is higher in patients with nodules located in the posterior and superior segments of the liver (segments IVb, VII, and VIII) *(52)*.

Results of a pretransplantation biopsy may help address the important issue of tumor differentiation. There is growing evidence that tumor grade has a marked effect on survival after both resection and liver transplantation *(53)*. The risk of recurrence is higher in patients with moderately or poorly differentiated tumors compared with those with well-differentiated tumors. Several series have shown that a small subset of patients with tumors outside the Milan criteria have an excellent outcome. It can be assumed that these patients have well-differentiated tumors and no vascular invasion. Hence, pretransplantation liver biopsy may be a useful tool for identifying patients who are outside the standard Milan criteria but may still be acceptable candidates for transplantation. In the future, molecular analysis of tissue samples may help identify patients at low risk of recurrence, which would further support the usefulness of pretransplantation biopsy.

5.2. Pitfalls of Liver Biopsy

Biopsy of small lesions (<2 cm) may not be reliable. When the lesion is so small needle placement may be difficult and one cannot be certain that the sample did indeed originate from the lesion. There is also a disagreement between pathologists as to the dividing line between dysplasia and well-differentiated HCC *(54)*. Finally, it may be difficult to distinguish well-differentiated HCC from normal liver on fine needle biopsy where architectural features of HCC, such as widened plates, might be lost.

Percutaneous biopsy of HCC carries a potential risk of tumor seeding along the needle tract. Months to years after a liver biopsy, some patients may be found with parietal tumor involving soft tissues, skin, peritoneum, and ribs. Rarely, there may be peritoneal dissemination distant from the site of puncture. Needle tract seeding can also occur in the post-transplantation

period, after the native liver has been removed. The overall risk of needle tract seeding in the largest series is <2% *(55)*. Risk factors for needle tract seeding have not been clearly identified. There is no evidence that the size of the needle, number of punctures, location of the tumor (sub-capsular), or poor differentiation represent important risk factors. One small study involving 32 patients suggested that the risk of seeding could be increased up to 12% after radiofrequency ablation due to the larger diameter of the needle *(56)*. However, increased risk has not been confirmed by another larger study which involved 1,314 patients undergoing radiofrequency ablation. The risk was <1% in the larger study *(57)*. Until now, there has not been clear evidence that pretransplantation biopsy increases the risk of post-transplantation recurrence, independent of needle tract seeding.

US-guided percutaneous liver biopsy also has several contraindications. These include ascites, low coagulation factors (INR > 1.7), low platelet count ($<50 \times 10^9$/L), or any condition that could cause an increase in bleeding. These occur mostly in patients with advanced cirrhosis and high MELD scores.

6. PRIMARY AND SECONDARY CHEMOPREVENTION OF HCC

The prognosis of HCC is very poor if diagnosed in the symptomatic stage. Most studies report a 5-year survival of less than 5% in symptomatic HCC. Primary HCC prevention includes universal vaccination for Hepatitis B, antiviral therapy of patients with chronic HBV or HCV, reduction in the amount of alcohol consumption, minimizing food contamination with aflatoxins, etc. For patients with genetic diseases such as precirrhotic hemochromatosis, there is a potential for HCC prevention by identifying affected family members at risk. Reduction of iron overload by phlebotomy in this selected group of patients has been shown to eliminate the progression of hemochromatosis and hence prevent cirrhosis and HCC. Preventative measures therefore should have a major impact on the incidence of HCC in patients with acquired and inherited liver disease. The prevention of local recurrence or the development of new HCC lesions in patients after successful surgical or non-surgical HCC treatment (secondary prevention) is of paramount importance and can significantly improve disease-free and overall patient survival.

6.1. Primary Prevention

In the setting of primary prevention the epidemiologic data available point to vaccination against HBV as the most efficient primary prevention measure currently available to reduce HCC incidence and mortality in the

high-incidence areas *(58)*. Eradication of HBV by vaccination has reduced the incidence of HCC in children in Taiwan *(59)*. Since there is no vaccine available for HCV, primary prevention of new infection should be the goal by rigorous implementation of infection control practices to prevent nosocomial and iatrogenic HCV transmission and secondary prevention of HCV transmission from infected persons to other persons through counseling *(60)*. Although screening of the whole population is not recommended, it is important to test for HCV in all "high-risk" individuals. Prevention of chronic liver disease of any etiology (alcohol, obesity) is essential to prevent HCC worldwide. Elimination of aflatoxin from the food supply in areas where agricultural products are stored under conditions that favor the growth of *Aspergillus flavus* and *Aspergillus parasiticus* is strongly needed. A recent case–control study from Sudan clearly shows that reduction of aflatoxin-contaminated foods may be a useful public health strategy in HCC prevention *(61)*.

6.2. Treatment of HBV and Prevention of HCC

While both viruses are linked to HCC, risk of cancer differs between HBV and HCV infection. About 10% of HBV-associated cancers occur in patients without cirrhosis, whereas HCC almost never occurs in the absence of cirrhosis in HCV infection. HCC risk is increased in those who are hepatitis B e antigen (HBeAg) positive and/or detectable HBV DNA. Recent data from a population-based prospective cohort study of greater than 3,500 patients, the REVEAL-HBV study from Taiwan, have shown that the progression to cirrhosis in HBV-infected patients is correlated strongly with the level of circulating virus *(62)*. From the same study, elevated serum HBV DNA level (>10,000 copies/mL) was shown to be a strong predictor of HCC, independent of HBeAg, serum alanine aminotransferase level, and liver cirrhosis *(63)*. The risk of HCC increases with the level of HBV DNA inferring that suppression of viral replication with antiviral therapy will decrease the risk of cancer. In one of the few prospective, randomized, controlled clinical trials involving patients with cirrhosis, lamivudine has been shown to reduce the development of complications of cirrhosis including HCC *(64)*. The risk of HCC was decreased in the treatment group vs. control group (3.9 vs. 7.4%; $P = 0.047$). This strongly suggests that there is a significant benefit of antiviral therapy in reducing risk of HCC development in patients with chronic HBV disease and significant fibrosis. The benefits of antiviral therapy are partially lost in those with ongoing replication due to either inadequate viral suppression or resistance. Hence, long-standing viral suppression may be required with other anti-HBV medications to prevent the development of HCC. These observations provide a rationale for potent,

long-lasting viral suppression, probably even beyond HBeAg seroconversion, that is likely achievable with currently available oral agents *(65)*.

6.3. Treatment of HCV and Prevention of HCC

Development of HCC occurs only in the setting of cirrhosis with HCV infection. Most data on treatment of these compensated cirrhotic patients are largely derived from subgroup analyses of clinical trials and have typically combined patients with bridging fibrosis and those with cirrhosis. These patients appear to respond less well to therapy than those with minimal or no fibrosis *(66)*. However, sustained virological response likely reduces but does not eliminate the risk of HCC. It is not known if there is a benefit in viral suppression in the absence of viral clearance of HCV infection. Potential secondary benefits of interferon provided the rationale for three large prospective studies (HALT-C, COPILOT, and EPIC3) examining the effects of low-dose pegylated interferon maintenance therapy in patients with advanced fibrosis or cirrhosis who had failed to clear the virus with initial standard treatment. Clinical endpoints include complications of portal hypertension and the development of HCC. Two of these studies are still ongoing; however, end-of-treatment data were recently presented from the HALT-C study where low-dose interferon was not shown to reduce the rate of disease progression and there was no significant difference in the incidence of HCC compared to placebo *(67)*.

In contrast, some studies from Asia and Europe have revealed promising results in terms of risk reduction for HCC. A recent multicenter nonrandomized study from Japan showed that patients with cirrhosis treated with interferon had a decreased risk of developing HCC compared to a group of untreated cirrhotic patients *(68)*. In a second study also from Japan, interferon monotherapy in cirrhotic patients led to a decrease in HCC *(69)*. Two other European studies have also suggested a benefit of interferon monotherapy in reducing risk of HCC by twofold *(70, 71)*. In summary, HCC risk is linked to fibrosis in many studies and antiviral therapy may improve fibrosis and thus also reduce HCC risk. Interferon may, in addition, have anticancer properties independent of its antiviral effects. We eagerly await final data from the other two US-based, large, prospective studies evaluating maintenance interferon therapy.

6.4. Secondary Prevention

There are little data about benefits of HBV or HCV antiviral therapy in reducing the risk of recurrent HCC after initial therapy for HCC (resection, radiofrequency ablation, or transarterial chemoembolization). Recently, new agents have been evaluated for adjuvant therapy of HCC

recurrence, including retinoids, intra-arterial I^{131}, adoptive immunotherapy, and interferon. A recent prospective randomized control trial from Taiwan showed no benefit of INF-α-2b in reducing risk *(72)*.

7. CONCLUSIONS

HCC is a neoplasm presenting many of the characteristics that would suggest efficacy of screening, as there is a well-defined population at risk; low cost, non-invasive diagnostic tools are available; and curative treatments exist which can provide excellent long-term survival. In this chapter, we have demonstrated that the clinician plays an integral role in instituting primary and secondary preventative measures in patients with chronic liver disease; in recognizing the various clinical manifestations of the disease; in meticulously screening the population at risk; and in directing the further evaluation of patients with positive diagnostic testing. Finally, it is of paramount importance to diagnose those patients at a stage where a curative approach can still be adopted rather than one of palliation.

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11 Screening and Biomarkers for Hepatocellular Carcinoma

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CONTENTS

ABSTRACT

Hepatocellular carcinoma (HCC) is currently the fifth most common tumor worldwide and is expected to continue to increase in incidence over the next couple of decades. The majority of patients with HCC have cirrhosis of the liver, with chronic hepatitis B and C as the major etiological agents. Despite advances in technology, the prognosis of patients with HCC has shown little improvement over time likely due to the fact that most patients are diagnosed at advanced stages. HCC meets the criteria established by the Word Health Organization for performing surveillance in those at risk for developing this tumor, i.e., patients with cirrhosis of the liver. The objective of surveillance is to use a relatively simple and inexpensive test in a large number of individuals to determine if they are likely or unlikely to have cancer, with an overall goal of reducing morbidity and mortality from the cancer. Alpha-fetoprotein and liver ultrasound are the most widely utilized surveillance tests but their performance is not optimal. There is an urgent

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need for new surveillance tests. In this chapter we will review the criteria and the current and newer tests for the surveillance of HCC.

Key Words: Hepatocellular carcinoma; surveillance; screening; biomarkers

1. INTRODUCTION

There have been little improvements in the overall survival of hepatocellular carcinoma (HCC) over the last decades primarily due to patients being diagnosed at advanced stages. One of the most important aspects of HCC is that it commonly occurs in patients with chronic liver disease, which also complicates the treatment of these patients. However, this important fact should be taken advantage in devising a strategy for the early detection of this tumor. In this chapter we will review the criteria for the screening or surveillance for HCC, the tests that are utilized, and new tests that may lead to better outcomes.

2. SCREENING/SURVEILLANCE FOR HEPATOCELLULAR CARCINOMA

The decision to screen an at-risk population for cancer is based on well-established criteria *(1)*. The objective of screening is the use of a relatively simple and inexpensive test in a large number of individuals to determine whether they are likely or unlikely to have the cancer for which they are being screened *(2)*. Screening is the one-time application of a test that allows detection of a disease at a stage where curative intervention may achieve the goal of reducing morbidity and mortality. Surveillance is the continuous monitoring of disease occurrence (using the screening test) within an at-risk population to accomplish the same goals of screening.

Criteria have been developed, first promoted by the World Health Organization, to assess the benefits of screening for a specific disease *(4)*: (a) the disease in question should be an important health problem; its significance may be defined by disease burden, including morbidity and mortality. (b) There should be an identifiable target population. (c) Treatment of occult disease (i.e., disease diagnosed before the symptoms appear) should offer advantages compared with the treatment of symptomatic disease. (d) A screening test should be affordable and provide benefits justifying its cost. (e) The test must be acceptable to the target population and to health-care professionals. (f) There must be standardized recall procedures.

(g) Screening tests must achieve an acceptable level of accuracy in the population undergoing screening. (h) Surveillance should reduce mortality from the disease. We will evaluate the rationale for the surveillance of patients with HCC based on these criteria.

2.1. The Disease in Question Should Be an Important Health Problem; Its Significance May Be Defined by Disease Burden, Including Morbidity and Mortality

HCC is the fifth most common tumor worldwide, with an incidence rate that is similar to the death rate. In the 2007 annual report to the nation on the status of cancer, liver cancer was the 13th most common tumor in the United States and it had the largest increase in incidence of all solid tumors when 1995 and 2004 were compared *(3)*. The incidence of HCC has been rising in both Europe and the United States, largely due to the growing prevalence of hepatitis C cirrhosis *(4–7)*. A molecular clock study indicated that the epidemic of hepatitis C (HCV) in the United States started in the 1960s and peaked in the late 1980s *(8)*. Because of the lag time between the onset of infection and the development of cirrhosis, the authors postulate that the incidence of HCV-related HCC will continue to increase over the next 20 years. HCC is the third most common cause of cancer-related deaths worldwide resulting in over 500,000 deaths per year. In the United States HCC is the eighth most common cause of cancer-related death at 8.5 deaths per 100,000 but has the largest increase in mortality of all solid tumors when comparing 2004 to 1995 *(3)*. Despite advances in technology and available treatments, there have been little improvements overall due to the fact that most patients are diagnosed at advanced stages *(9*, *10)*. Together with the increasing incidence, it may lead to a significant health burden.

2.2. There Should Be an Identifiable Target Population

Cirrhosis has been recognized as the most important risk factor for the development of HCC *(11)*. Table 1 shows the incidence rates for those with HCV, hepatitis B (HBV), and alcoholic-related cirrhosis *(12)*. This table shows that HCV and HBV are the major etiological agents that lead to the development of HCC while alcohol does increase the risk to a lesser degree. HCV-associated cirrhosis is the causative agent that has been largely responsible for the increase in incidence of HCC in the United States *(13)*. However, HBV is the leading cause of HCC worldwide, particularly in Asia and Africa *(14)*. Recently, an association between non-alcoholic liver disease and HCC has been made *(15)*, but there are no prospective cohort studies evaluating the natural history of non-alcoholic fatty liver disease. Other etiologies of chronic liver disease such as hemochromatosis, primary

Table 1
Overall Hepatocellular Carcinoma Incidence Rates According to Etiology of Liver Disease

Disease	*Location*	*No. patients*	*Follow-up (years)*	*HCC incidence**	*95% CI*
Hepatitis C	Europe/USA	1284	4.5	3.7	3.2–4.1
	Japan	626	5.8	7.1	6.1–7.9
Hepatitis B	Europe	401	5.8	2.2	1.6–2.8
	Taiwan	278	4.3	3.2	1.9–4.5
	Japan	306	5.8	4.3	3.4–5.2
Alcohol	Europe	584	5	1.7	1.2–2.1
	Japan	174	4.5	1.8	0.8–2.7

CI= confidence intervals. * Incidence per 100 person-years. Modified from Fattovich et al. *(12).*

biliary cirrhosis, autoimmune hepatitis, and alpha-1 antitrypsin deficiency are less common causes of chronic liver disease with prevalence rates in patients with HCC between 1 and 8% *(16–18)*. Furthermore, improvements in the survival of patients with cirrhosis due to better specialty care may further increase the number of individuals at risk for developing HCC *(19)*. At the present time, patients with cirrhosis, regardless of the etiology, should undergo surveillance for HCC *(20)*.

Even though the annual risk of developing HCC among patients with cirrhosis is between 2 and 7%, not every patient with cirrhosis will develop this tumor. Male gender, older age, obesity, alcohol and tobacco consumption, and diabetes are factors associated with an increased risk of HCC *(21–25)*. In patients with chronic HBV infection, a baseline HBV DNA level of greater than 100,000 copies/mL increases the risk of HCC 10-fold *(26)*. This biological gradient of HCC risk in relation to HBV DNA levels suggests that persistent viral replication increases the risk of HCC. A prospective cohort study of patients with cirrhosis found that prothrombin activity <75% of baseline, age >55, platelet count <75 mm^3, and HCV were independent risk factors for developing HCC *(27)*. They stratified patients into a high-risk group (presence of these factors) and into a low-risk group (absence of risk factors), and the 5-year cumulative incidence of HCC was 30% for the high-risk group and 4% for the low-risk group ($p < 0.0001$). Further studies should be performed to determine if stratification according to risk factors is beneficial for delineating a sub-group of patients with cirrhosis that may be at a higher risk of developing HCC in whom more aggressive surveillance can be applied.

2.3. Treatment of Occult Disease Should Offer Advantages Compared with the Treatment of Symptomatic Disease

The effectiveness of the treatments for HCC will depend on the stage at the time of diagnosis. For early-stage tumors, surgical resection has provided 5-year survival rates of 70% in carefully selected patients with preserved hepatic function, no evidence of portal hypertension, and single small asymptomatic tumors (<5 cm in maximal diameter) *(20)*. Liver transplantation is the preferred method of treatment for patients not amenable to surgical resection but for those restricted to the Milan criteria (single nodule <5 cm or <3 nodules each <3 cm in diameter) *(28)*. The 5-year survival reported for liver transplantation is >70% *(29)*. Ablative treatments, specifically percutaneous ethanol injection and radiofrequency ablation, have 5-year survival rates similar to hepatic resection *(30)*. Therefore, therapies currently exist for patients with early-stage HCC, and an efficacious surveillance program is critical for the identification of HCC at these early stages.

2.4. A Screening Test Should Be Affordable and Provide Benefits Justifying Its Cost

The standard threshold for cost-effectiveness of a medical test or procedure has been determined to be a maximal of $50,000 per quality-adjusted life year (QALY). Economic models studying the benefits of surveillance programs in HCC have been performed. Surveillance with biannual alpha-fetoprotein (AFP) and ultrasonography in Child class A cirrhotics increase the mean life expectancy with cost-effectiveness ratios between $26,000 and $55,000 per QALY *(31)*. When a similar analysis was performed in HCV cirrhotics, the cost-utility ratio was $26,689 per QALY *(32)*. Another study evaluating the cost-effectiveness of biannual AFP and ultrasound in HCV Child class A cirrhosis showed a cost-effective ratio of $33,083 per QALY *(33)*. Therefore, screening with ultrasound and AFP has been shown to be cost-effective in compensated cirrhotics even though the performance of these tests is not the best.

2.5. The Test Must Be Acceptable to the Target Population and to Health-Care Professionals

Surveillance for HCC seems to be acceptable to patients with cirrhosis. Such data come indirectly from cohort studies showing that only about 3–18% of cirrhotic patients were noncompliant with surveillance using ultrasound and AFP *(11)*, which compares favorably with the 67%

noncompliance rate seen with using colonoscopy for colon cancer screening *(34)*. HCC surveillance also seems to be well accepted by physicians. In a national survey of 554 members of the American Association for the Study of Liver Disease, 84% of respondents indicated that they routinely screened patients with cirrhosis for HCC using AFP and ultrasound *(35)*.

2.6. There Must Be Standardized Recall Procedures

A recent consensus conference offered guidelines on how to investigate abnormalities of the commonly used screening tests (AFP and ultrasound) in patients with cirrhosis *(20)*. CT scan, MRI, and contrast-enhanced ultrasound are the major diagnostic modalities used to establish the diagnosis of HCC without the need for a histopathological examination. The main imaging characteristic for HCC is the finding of arterial enhancement of the lesion followed by washout of contrast in the delayed venous phases *(36)*. A recent study has validated the American Association for the Study of Liver Disease guidelines for the diagnostic evaluation of an abnormal surveillance test *(37)*. Therefore, appropriate recall modalities do exist to evaluate abnormal surveillance tests.

2.7. Screening Tests Must Achieve an Acceptable Level of Accuracy in the Population Undergoing Screening

Ultrasound and AFP have been recommended as the primary radiologic screening test for HCC *(20)*. US is inexpensive, non-invasive, and widely available, which makes it an attractive surveillance test. There have been no randomized controlled trials in patients with cirrhosis to date assessing the efficacy of US as a surveillance test. The performance of ultrasound has been evaluated primarily in cohort studies as shown in Table 2 *(38–46)*. The sensitivity for the detection of early-stage HCC ranges from 25 to 100%, while the specificity ranges from 82 to 100%. The high degree of operator dependence, differences in the equipment, body habitus, and the lack of evidence by randomized trials are significant limitations of US as a surveillance test for HCC.

AFP has been the most widely utilized serologic test to screen for HCC. The operating characteristics of AFP are dependent on the cutoff level chosen to support the diagnosis of HCC. At higher cutoff levels, the test is more specific for HCC but at a cost of decreased sensitivity; at low cutoff levels conversely, AFP becomes increasingly sensitive but with a higher rate of false positives *(47)*. In a case–control study using 170 patients with

Table 2
The Performance Characteristics of Ultrasonography in Cohort Studies for the Detection of HCC

Author	*Cohort*	*No. of total HCC cases*	*No. of early HCC cases*	*Sensitivity (%)*	*Specificity (%)*
Cottone *(38)*	Childs A	5	4	80	NA
Pateron *(39)*	Childs A–B	14	5	21	82
Bolondi *(40)*	Childs A–B	61	50	75	95
Kobayashi *(41)*	Cirrhosis	8	6	50	98
Sheu *(42)*	Cirrhosis	7	7	100	100
Oka *(43)*	Cirrhosis	40	33	68	NA
Henrion (44)	Cirrhosis	6	6	83	NA
Zoli *(45)*	Cirrhosis	34	32	94	NA
Santagostino *(46)*	Cirrhosis	8	2	25	NA

NA = not available.

HCC, about 60% of the patients had advanced HCC, and 170 matched patients without HCC demonstrated that the optimal cutoff was 20 ng/mL via receiver operating curve analysis *(48)*. Therefore, a level greater than 20 ng/mL is the most commonly used cutoff in clinical practice to trigger a recall test for the diagnosis of HCC. Even at the optimal cutoff level in this study, the sensitivity was only 60% while the specificity was 90%. A recent systematic review of five studies evaluating AFP in patients with hepatitis C cirrhosis showed sensitivities ranging from 41 to 65% and specificity ranging from 80 to 94% *(49)*. In addition, serum AFP values are frequently elevated among patients with chronic hepatitis C with advanced hepatic fibrosis even in the absence of HCC, with levels declining after antiviral therapy *(50)*. AFP alone is insufficient for the surveillance for HCC among patients with cirrhosis. In hepatitis B carriers, the combination of ultrasound and AFP increased the sensitivity of HCC detection when compared to either test alone, increasing from 71% with ultrasound alone to 79% when ultrasound and AFP were used together *(51)*. Chronic elevations of AFP have also been shown to increase the risk of developing HCC among patients with cirrhosis *(52)* and in hepatitis B carriers *(53)*. While better tests are needed to improve the detection of early-stage HCC, AFP offers benefits in the surveillance of patients with cirrhosis leading to diagnosis in about half of the patients with HCC and determining their risk of developing this tumor.

3. EFFICACY OF SURVEILLANCE

As previously indicated, the goal of a surveillance program is for the tests to reduce overall mortality. The most reliable method to evaluate the efficacy of ultrasound and AFP for HCC surveillance would be a randomized controlled trial. There have been two large randomized controlled trials conducted in China using ultrasound and AFP among patients with chronic hepatitis B *(54, 55)*. In both trials, surveillance was conducted every 6 months and compared to patients who did not receive any routine screening. The first study evaluated 17,920 patients that are carriers of the hepatitis B virus who were randomized to surveillance ($n = 8{,}109$) or no surveillance ($n = 9{,}711$) and then followed for an average of 14.4 months *(54)*. Of the patients randomized to the surveillance group, 38 patients developed HCC of whom 29 (76.3%) were detected at early stages, whereas 18 patients developed HCC in the no-surveillance group, of whom none were detected at an early stage ($p < 0.01$). A higher proportion of patients in the surveillance group met criteria for surgical therapy, with 24 patients having surgical resection in the surveillance group compared to zero patients in the no-screening group ($p < 0.05$). Accordingly, the 1-year and 2-year survival rates for the surveillance group were 88.1 and 77.5%, respectively, compared to a 0% survival rate at 1 year for the no-screening group. The authors concluded that surveillance reduces HCC-associated mortality. The second randomized controlled trial evaluated 19,200 hepatitis B carriers who were randomized to surveillance ($n = 9{,}757$) and no surveillance ($n = 9{,}443$) *(55)*. A total of 86 patients developed HCC in the surveillance group, of which 45% were early stage, compared to 67 patients with HCC in the no-surveillance group, of which none were early stage. Table 3 summarizes the results. The mortality rate of patients undergoing surveillance was significantly lower than the control group (83.2 vs. 131.5 per 100,000, $p < 0.01$), with a hazard ratio of 0.63 (95% CI 0.41–0.98). These results demonstrate that the strategy of surveillance with US and AFP among patients with chronic hepatitis B reduces overall mortality. However, it is unclear if all the patients in these two studies had the same risk of developing HCC, given the low rate of development of HCC seen. These studies did not mention the number of patients that had cirrhosis or evidence of viral replication and most likely had patients that were asymptomatic carriers, which are at a lower risk for developing HCC. Therefore, the results are not generalizable to the majority of patients at risk for developing HCC.

While randomized controlled trials have been performed in China using patients with chronic hepatitis B, the results cannot be extrapolated to cirrhotic patients, who account for the majority of patients with HCC. No randomized trials have been performed in a cirrhotic population, so most of the data on surveillance in patients with cirrhosis come from cohort studies. Some studies have shown that patients undergoing surveillance

Table 3
Stage Distribution, Treatment, and Survival of Patients with HCC in the Surveillance and Control Groups

	Surveillance group (n = 86)	*Control group (n = 67)*
Stage[a]		
I	52 (60%)	0 (0%)
II	12 (14%)	25 (37%)
III	22 (26%)	42 (63%)
Treatment		
Resection	40 (47%)	5 (7%)
TACE/PEI	28 (32%)	28 (42%)
Symptomatic	18 (21%)	34 (51%)
Survival (%)[b]		
1 year	65.9	31.2
3 years	52.6	7.2
5 years	46.4	0

Adapted from reference *(55)*. [a] Chi square = 61.4, $p < 0.01$. [b]log-rank = 35.5, $p < 0.01$.

with ultrasound and AFP have a better overall survival when compared to either historical controls or patients with HCC who did not undergo surveillance. Table 4 shows the details of these cohort studies including the number of HCC and early-stage HCC that developed during follow-up *(38–46, 56–67)*. The results of these studies are also fraught with lead-time and length–time biases that limit their generalizability of improvements in survival with surveillance. Therefore, the impact of surveillance on mortality in patients with cirrhosis has only been assessed in non-randomized trials to date. As shown in Table 3, there has been a significant amount of heterogeneity among these studies pertaining to the sample size (ranging from 66 to 1,599), population studied (Child class A, Child class A or B, transplant candidates), the incidence of HCC (ranging from 3 to 28%), and number of early-stage HCC detected (ranging from 24 to 100%). Randomized or better controlled trials are needed in this area.

4. NOVEL BIOMARKERS

The ideal marker for HCC would be specific for HCC and not be detected in pre-malignant liver disease (i.e., cirrhosis regardless of the etiology). It should be easily accessed, easily measurable, reproducible, minimally

Table 4
Cohort Studies in Patients with Cirrhosis Evaluating Ultrasound and AFP for the Detection of Hepatocellular Carcinoma

Author	*No. of patients*	*Mean follow-up (months)*	*Surveillance method*	*HCC detected n (%)*	*Early-stage HCC n (%)*
Cottone *(38)*	147	24	AFP and ultrasound	5 (3)	4 (80)
Pateron *(39)*	118	36	AFP, DCP, and ultrasound	14 (12)	5 (36)
Bolondi *(40)*	313	56	AFP and ultrasound	57 (18)	53 (87)
Kobayashi *(41)*	95	50	AFP, ultrasound, and CT	8 (8)	6 (75)
Sheu *(42)*	223	17	AFP and ultrasound	7 (3)	7 (100)
Oka *(43)*	140	41	AFP and ultrasound	39 (28)	27 (82)
Henrion *(44)*	94	34	AFP and ultrasound	6 (6)	5 (83)
Zoli *(45)*	164	28	AFP and ultrasound	34 (21)	32 (94)
Santagostino *(46)*	66	72	AFP and ultrasound	8 (12)	2 (25)
Velazquez *(56)*	463	39	AFP and ultrasound	38 (8)	18 (47)
Sangiovanni *(57)*	417	148	AFP and ultrasound	112 (27)	27 (24)
Tradati *(58)*	40	48	AFP and ultrasound	6 (15)	2 (33)
Van Thiel *(59)*	100		AFP, ultrasound, and triple-phase CT	14 (14)	13 (93)
Imberti *(60)*	228	44	AFP and ultrasound	38 (17)	14 (37)
Colombo *(61)*	417	33	AFP and ultrasound	26 (6)	9 (35)
Cottone *(62)*	147	65	AFP and ultrasound	30 (20)	25 (83)
Degos *(63)*	416	68	AFP and ultrasound	60 (14)	37 (62)

(Continued)

Table 4
(Continued)

Author	*No. of patients*	*Mean follow-up (months)*	*Surveillance method*	*HCC detected n (%)*	*Early-stage HCC n (%)*
Bruno *(64)*	163	68	AFP and ultrasound	22 (13)	16 (73)
Caturelli *(65)*	1599	43	AFP and ultrasound	269 (17)	253 (94)
Tong *(66)*	173	35	AFP and ultrasound	31 (18)	18 (58)
Iavarone *(67)*	201	50	AFP and ultrasound	27 (13)	17 (63)

AFP = alpha-fetoprotein; DCP = des-gamma carboxy-prothrombin; HCC = hepatocellular carcinoma

invasive, accurate, and acceptable to patients and physicians *(68)*. The current tests do not meet these criteria and new ones are needed. The recent developments of gene-expression microarrays, proteomics, and tumor immunology permit thousands of genes and proteins to be screened simultaneously. With the growing application of these techniques, it is anticipated that there will be an explosion of new biomarkers for cancer screening including HCC in the next decade. To establish a formal framework to guide the process of biomarker evaluation and development, a five-phase program is utilized by the Early Detection Research Network (EDRN) of the National Cancer Institute (Table 5) *(69)*. These five phases help define criteria to determine the current status of biomarkers in the published literature, to assess how close these biomarkers are to clinical application, and to serve as a guide for future biomarker development. Table 6 shows promising biomarkers for HCC and level of evidence according to the phases of biomarker development.

4.1. Des-Gamma Carboxy-Prothrombin (DCP)

DCP is an abnormal prothrombin protein that is generated as a result of an acquired defect in the posttranslational carboxylation of the prothrombin precursor in malignant hepatic cells *(70)*. A single center case-controlled study showed that DCP was more sensitive and specific than total AFP *(71)*. Several prospective cohort studies in patients with cirrhosis without HCC have been performed to determine the performance of DCP *(72–75)*. The sensitivities for detecting HCC ranged from 23 to 57% compared to 14 to

Table 5
Phases of Biomarker Validation in Cancer Surveillance Studies

Phases	*Type of study*	*Aims*
1	Preclinical exploratory	Promising markers identified
2	Clinical assay and validation	Assay detects established disease
3	Retrospective longitudinal	Biomarker detects preclinical disease
4	Prospective screening	Confirm ability of marker to detect early-stage disease
5	Cancer control	Impact of screening on reducing tumor burden in at-risk population

71% for AFP. In the largest study on DCP, 734 patients with cirrhosis were followed for a mean of 13 months (range 7–17 months) during which HCC was detected in 29 patients. The sensitivity and specificity of DCP at baseline was 41% and 90%, and 40% and 62% for AFP, respectively. Overall, AFP and DCP had equal sensitivity but DCP had better specificity. Large studies are underway to evaluate the role of DCP in the detection of early-stage HCC.

4.2. Lens Culinaris Agglutinin Reactive Fraction of AFP (AFP-L3)

Lens culinaris agglutinin is a plant-derived lectin that recognizes fucose residues on *N*-glycosylated polypeptides *(68)*. Several variants of AFP with differences in lectin affinities have been identified. One variant, the fucosylated variant, has a high affinity of the sugar chain to *lens culinaris*. This variant has been shown to be more specific for HCC than total AFP *(76)*. Prospective studies in patients with cirrhosis have shown sensitivities for AFP-L3 ranging from 55 to 75% and specificities from 68 to 90% *(77–79)*. However, two studies included only HCC patients with elevated total AFP at baseline making it impossible to compare the accuracy of AFP-L3 with total AFP. A prospective study evaluated the clinical utility of AFP-L3 in a North American multicenter cohort *(80)*. The authors evaluated 332 patients with HCV cirrhosis and 34 developed HCC. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for AFP (cutoff > 20 ng/mL) were 61, 71, 34, and 88%, respectively, while for AFP-L3 (cutoff 10%) were 36, 91, 51, and 85%, respectively. The main utility of AFP-L3% was that in someone with cirrhosis it increases the risk of developing HCC. At this time there is no evidence of AFP-L3's efficacy in the

Table 6
Promising Biomarkers for the Detection of Hepatocellular Carcinoma According to the Phases of Biomarker Development

Biomarkers	*Biological material*	*Level of evidence*
Glypican-3	Tissue Serum	Phase 1
Golgi protein 73	Serum	Phase 1
p16 Methylation	Serum	Phase 1
Human hepatocyte growth factor	Serum	Phase 1
Des-gamma carboxy-prothrombin	Serum	Phases 1 and 2
AFP-L3	Serum	Phases 1, 2, and 3
Ctokeratin-19	Serum	Phase 1
90 K/MAC-2BP glycoprotein	Serum	Phase 1
Transforming growth factor-beta 1	Serum	Phase 1
Lipoprotein (a)	Serum	Phase 1
Erythrocyte-binding polyamine	Serum	Phase 1
Tissue polypeptide-specific antigen	Serum	Phase 1
C-reactive protein	Serum	Phase 1
Squamous cell carcinoma antigen	Serum	Phases 1 and 2
Osteopontin	Plasma	Phase 1
p53 antibodies	Serum	Phase 1
CD24 gene	Tissue	Phase 1
Telomerase activity	Tissue	Phase 1
Prothymosin alpha	Tissue	Phase 1
Microsatellite DNA analysis	Tissue	Phase 1
HCC-associated gene 1	Tissue	Phase 1
Hepatoma-specific gamma-glutamyltransferase	Tissue	Phase 1

surveillance of patients with cirrhosis or that these are better than AFP and ultrasound in this capacity.

4.3. Glypican-3

Glypican 3 is a member of the glypican family of cell-surface heparan sulfate proteoglycans, recently found to be upregulated in early-stage HCC compared to normal hepatic tissue *(81)*. Evaluation of glypican-3 as a serum marker for HCC has been reported *(82)*. In this study, glypican-3 expression in liver was detected using immunohistochemistry in 0/22 cirrhotics without dysplasia or HCC, 1/5 cirrhotics with high-grade dysplasia, and 21/29 HCCs. For tumors <3 cm, glypican-3 expression was detected in 11/11

and AFP in only 2/9. Using enzyme-linked immunoassay, glypican-3 was detected in the serum from 18/34 (53%) patients with HCC and only 1/20 (5%) patients with cirrhosis ($p = 0.0049$). More recently, it was found that glypican-3 expression was an independent histological marker for differentiating early HCC from cirrhosis *(83)*. Further studies are needed to determine if the sensitivities can be improved in the serum in order for glypican-3 to be utilized in the surveillance for HCC.

4.4. Golgi Protein (GP73)

GP73 is a resident Golgi protein that is upregulated in virus-infected hepatocytes *(84)*. Using Western blot assay, GP73 has been detected in serum with significantly higher levels among cirrhotics and patients with HCC than in normal subjects and patients with chronic hepatitis. In a phase 2 study, a total of 296 patients (152 cirrhosis controls and 144 HCC cases) were studied *(85)*. Serum GP73 levels were significantly higher in patients with HCC compared to those with cirrhosis ($p < 0.001$). GP73 had a sensitivity of 69% and a specificity of 75% at the optimal cutoff point of 10 relative units, with an area under the receiver operating curve of 0.79 vs. 0.61 for AFP ($p = 0.001$). GP73 levels had significantly higher sensitivity (62%) than AFP (25%) for diagnosing early HCC ($p < 0.0001$). Moreover, GP73 levels were elevated in the serum of 57% (32/56) individuals with HCC who had serum AFP levels less than 20 ng/mL. GP73 should be tested in a larger sample set to determine the performance characteristics.

4.5. Glycoproteins

The fucome is the subset of polypeptides that contain the sugar fucose. Fucosylated *N*-glycans derived from glycoproteins in the serum of patients with HCC are greatly elevated compared to healthy individuals, and a recent study showed more than 50 fucosylated serum proteins in the woodchuck model of HCC and in human HCC *(86)*. Fucosylated GP73 and hemopexin are examples of cases in which the measurement of these glycoproteins had sensitivities over 90% and was better than measuring the total amount. This is an interesting area of research that may lead to a significant biomarker but more validation studies are needed.

4.6. Human Hepatocyte Growth Factor (HHGF)

HHGF is a growth factor that has mitogenic, anti-apoptotic, and antifibrotic effects, and therefore, it is important in hepatocarcinogenesis. A recent study evaluated 70 patients with HCV cirrhosis and 38 patients with HCC in order to evaluate the role of HHGF in liver cancer *(87)*. In patients with HCC, however, HGF showed little localization in cancer cells, but was

noted in infiltrating mesenchymal cells in both cancerous and noncancerous regions, perhaps a measure of metastatic spread. Another study evaluated HHGF in 134 patients with HCV-related disease (62 had cirrhosis and 72 chronic hepatitis) who were followed for 4 years, 28 developing HCC *(88)*. Human HGF had a sensitivity and specificity of 100 and 63%, respectively, at the time of HCC diagnosis. These results are preliminary and require further study but the high sensitivity is promising for HHGF being a surveillance test.

4.7. Insulin Growth Factor-1 (IGF-1)

Deregulation of the insulin-like growth factor (IGF) axis, including the autocrine production of IGFs, IGF-binding proteins (IGFBPs), IGFBP proteases, and the expression of the IGF receptors, has been identified in the development of hepatocellular carcinoma (HCC). IGF-1 was measured in 114 patients with HCV-related cirrhosis followed for a mean of 56±12 months; AFP and ultrasound were monitored annually *(89)*. HCC developed in 20 patients. Among those in whom HCC developed, there was a mean annual decrease of 16 μg/L in IGF-1 levels until the diagnosis of HCC. A decrease in IGF-1 levels of 9.3 μg/L had a sensitivity of 70% for diagnosis of HCC. This is a well-done study that showed reductions in IGF-1 levels prior to the diagnosis of HCC. This marker should undergo further study as a HCC surveillance test.

4.8. Squamous Cellular Carcinoma Antigen (SCCA)

SSCA is a serine protease inhibitor physiologically present in the skin, which has been detected in HCC tissue *(90)*. SCCA is strongly expressed in HCC than peritumoral tissue, and it also increases the AFP diagnostic capability up to 90% *(91)*. A total of 961 patients, diagnosed as LC (462) and HCC (499), were enrolled to evaluate for the performance of SSCA *(92)*. The SCCA AUC was 0.656 (95% CI 0.625–0.686), and the cutoff value was 3.8 ng/mL, showing 41.9% sensitivity and 82.6% specificity. SSCA was complementary to AFP improving the sensitivity to 80%. A large study is underway to investigate this marker in HCC.

4.9. Osteopontin (OPN)

OPN is a highly phosphorylated and glycosylated protein, the modification after transcription is very important to its function. In hepatocellular carcinoma (HCC), the elevated expression of OPN at mRNA levels and its relationship with metastasis and poorer prognosis of the patients have been reported. OPN in HBV-related HCC was studied recently *(93)*. Thirty-nine of 72 (54.17%) HBV-related HCC specimens were positive for OPN with

cytoplasmic staining. OPN was highly expressed in the specimens with capsular infiltration compared to those without ($p < 0.05$) and also was significantly related with portal vein invasion ($p < 0.01$) and lymph node invasion ($p < 0.01$). In another study of 62 HCC patients, 60 patients with chronic liver diseases, and in 60 healthy controls, OPN was measured in the plasma *(94)*. Plasma OPN levels in the HCC patients (median 954 ng/mL, range 168–5,742) were significantly higher (p-value < 0.001) than those patients with chronic liver diseases (381 ng/mL, 29–1,688) or of a healthy control group (155 ng/mL, 10–766). Within the HCC patient group, plasma OPN level increased significantly with advancing degree of Child–Pugh class and of tumor stage. Diagnostic sensitivity and specificity of OPN for HCC was 87 and 82%, respectively (cutoff value: 617.6 ng/mL). OPN had a greater area under curve value (0.898) than AFP (0.745) or DCP (0.578), suggesting superior diagnostic accuracy of OPN. This marker has potential and should be studied further in larger trials.

4.10. Proteomics

Proteomics studies the complete set of proteins expressed in a given cell, tissue, or biofluid. Proteomics not only characterizes protein expression profiles but also identifies protein structures, localizations, activities, modifications, and interactions in physiological or pathological states. As proteins perform most biological functions, proteomics bridges the gap between the information coded in the genome sequence and the cellular behavior. Proteomics studies of HCC may not only elucidate the mechanisms of HCC initiation and progression but also have the potential to discover novel diagnostic and prognostic biomarkers as well as therapeutic targets. There are several techniques that can be applied to study the proteins and these include two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and surface-enhanced laser desorption/ionization (SELDI), which combines purification of samples on a wide variety of affinity matrices and identification by time-of-flight mass spectrometry (TOF-MS) *(95)*. However, these do not identify the proteins. Mass spectrometry is the current method of choice for the identification of proteins, as this method offers high analytical sensitivity and the capacity for high-throughput protein identification. Current studies have shown various patterns that appear to differentiate HCC from controls *(96)*, but these studies are in their infancy and prospective studies are required.

A recent study showed the potential of the proteomic approach. A total of 10 HCC tissues from patients with HCV cirrhosis were analyzed by 2D-PAGE *(97)*. Forty-seven protein spots that showed reproducible variation were identified by mass spectrometry, corresponding to 23 distinct genes. A positive correlation between transcript and protein level variations was

observed for only 7 out of the 23 genes. Proteolytic cleavage accounted for the discrepancies between messenger RNA and protein level changes for seven genes including calreticulin, protein disulfide isomerase (PDIA3), among others. Calreticulin and PDIA3 cleavage products were detected in sera of patients with HCC. A statistically high significant difference in calreticulin and PDIA3 fragment serum levels between patients with HCC and healthy individuals was observed. Amounts of calreticulin and PDIA3 fragments were also significantly different between patients with HCC and at-risk patients (patients with cirrhosis). This showed that isoforms or cleavaged proteins may become markers for HCC.

More sensitive and specific biomarkers for HCC are urgently needed. As we have discussed in this section, there are several biomarkers that appear interesting and requires further testing because most of these have been tested in phase 1 studies. It is unlikely that one biomarker will be sufficient and more likely it will be a panel of markers. With modern advances in the study of proteins, glycoproteins, and genes, it is likely that a panel of markers may soon be identified for the early detection of HCC. For now, AFP and US are currently the best surveillance tests for HCC.

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12 Use of Imaging Techniques to Screen for Hepatocellular Carcinoma

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CONTENTS

ABSTRACT

This chapter is intended to provide a state-of-the-art overview of liver imaging with emphasis on the roles of sonography, CT, and MRI in the cirrhotic patient. Included are the fundamentals of using imaging in a surveillance program to monitor the progression of cirrhosis and to detect hepatocellular carcinoma (HCC). Imaging features are discussed that are useful in distinguishing among the various focal lesions that may be found in the cirrhotic liver, including regenerative, dysplastic, and malignant nodules.

Key Words: CT; MRI; regenerative nodule; dysplastic nodule; hepatocellular carcinoma

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Accurate detection, characterization, and staging of hepatocellular carcinoma (HCC) are among the most difficult challenges facing radiologists and other physicians caring for patients with chronic liver disease. Most HCCs occur within the cirrhotic liver and the diffuse and focal abnormalities that characterize the cirrhotic liver are often difficult to differentiate by any imaging test. Nevertheless, cross-sectional imaging modalities (sonography, computed tomography, and magnetic resonance imaging) are applied frequently in the evaluation and surveillance of patients with chronic liver disease and much has been learned about the relative merits and accuracy of these tools. There are substantial variations among investigations in their recommendations for the choice and timing of imaging studies, many of which reflect the relative geographic prevalence of HCC and the availability and expense of imaging tests, as well as the enthusiasm and expertise of the interpreting physicians. In this chapter we will review the current knowledge and published recommendations for imaging surveillance of chronic liver disease and will present our own approach at the University of Pittsburgh Medical Center.

1. MONITORING THE CIRRHOTIC PATIENT

A variety of clinical and biochemical parameters are used to follow the progression of cirrhosis, including serum tests of liver function and tumor markers, such as α fetoprotein (AFP) and PIVKA II (protein induced by vitamin K absence or antagonist). The role of imaging is to measure and characterize the morphologic manifestations of cirrhosis (liver size, scarring, etc.), evaluate the hepatic and extrahepatic vasculature, assess the effects of portal hypertension, and detect and characterize focal hepatic masses.

2. FOCAL LESIONS IN THE CIRRHOTIC LIVER

2.1. Fibrosis

Fibrosis is present in all cirrhotic livers but uncommonly is visualized as a discrete structure on cross-sectional imaging. Fibrosis imparts the coarse, heterogeneous echo pattern that is the typical ultrasound appearance of the cirrhotic liver. When fibrosis forms thick septa or a confluent mass it is detectable by CT or MR. Confluent fibrosis can be mistaken for a mass lesion *(1,2)*, but has a characteristic set of features that allow confident diagnosis in most cases. On unenhanced CT it is hypodense to liver. On contrast-enhanced CT the fibrotic area shows progressive and prolonged enhancement and evidence of volume loss of the affected part of the liver, resulting in crowded vessels and hepatic capsular retraction (Fig. 1). MR shows similar morphologic features, including delayed persistent enhancement with IV

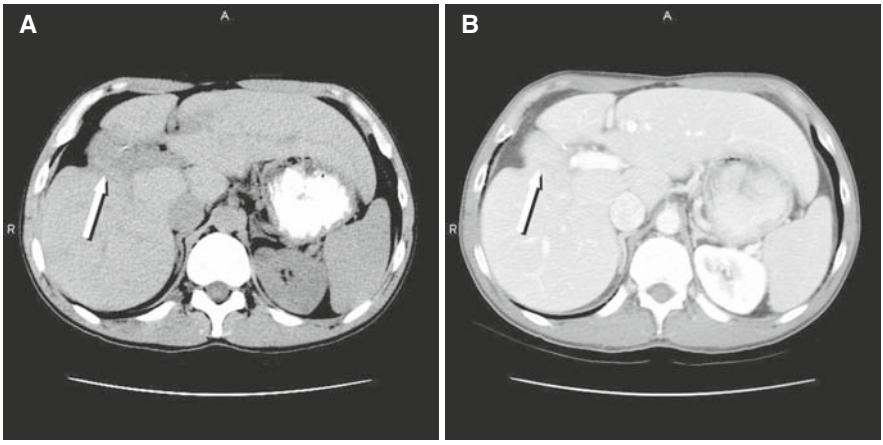

Fig. 1. Confluent hepatic fibrosis. (**A**) Unenhanced CT shows a hypodense lesion (*arrow*) bridging the anterior and medial segments of the liver. (**B**) Portal venous phase image shows iso-density to the corresponding area (*arrow*). Note the overlying retraction of the hepatic capsule indicating volume loss of this part of the liver. The lesion was isodense to the liver (*invisible*) on enhanced CT scans.

gadolinium contrast material. More intense enhancement on arterial or portal venous phase images (CT or MR) may make it difficult to distinguish confluent fibrosis from an infiltrative neoplasm such as HCC or cholangiocarcinoma.

2.2. Regenerating Nodules

The regenerating nodules of the cirrhotic liver include macronodular (typical in chronic hepatitis B) and micronodular lesions (more common in other causes of cirrhosis). Most regenerating nodules are not detected as discrete masses by cross-sectional imaging because they are too small or are too similar to surrounding liver parenchyma in terms of echogenicity (ultrasound), density or attenuation (CT), or intensity (MR).

Ultrasound may suggest a regenerating nodule as a relatively hypoechoic lesion relative to the surrounding hyperechoic fibrotic cirrhotic liver; however, ultrasound cannot distinguish accurately between regenerating nodules and malignant masses. Almost all sonographically detected focal hepatic lesions within a cirrhotic liver require further evaluation by CT or MR and/or percutaneous image-guided biopsy.

CT detects regenerating nodules when they are surrounded by fibrosis (with the fibrotic bands being hypodense on unenhanced CT) or when they contain iron deposits, so-called siderotic nodules. Regenerating nodules are typically hyperdense to liver on nonenhanced CT and are isodense to liver

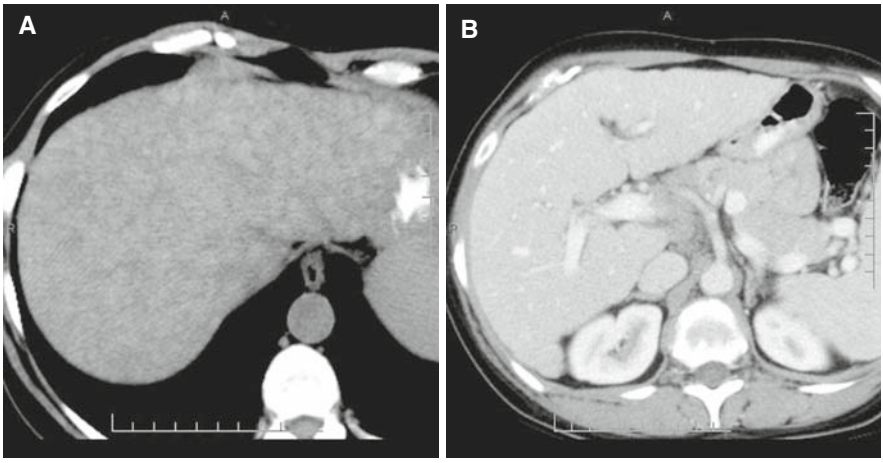

Fig. 2. Regenerating nodules. (**A**) Unenhanced CT demonstrates dozens of hyperdense rounded lesions throughout the liver. Most are about 1 cm in diameter. (**B**) Enhanced CT (portal venous phase). The nodules become isodense with the liver and cannot be detected.

(undetectable) on hepatic arterial phase and portal venous phase CT images *(3)* (Fig. 2).

MR detects more regenerating nodules than CT, though it too may depict only the larger or more siderotic nodules. Most regenerating nodules are isointense to liver on both T1- and T2-weighted images. Siderotic nodules have characteristic imaging features including decreased signal intensity on T2-weighted pulse sequences and "blooming" (appearing larger and more prominent) on gradient echo sequences with longer echo times *(4)* (Fig. 3).

Regenerating nodules usually enhance to the same or a lesser degree than the surrounding liver, a feature that makes them less apparent on contrast-enhanced CT or MR exams, but which serves as a useful distinguishing feature from other focal lesions. Some cirrhotic nodules, however, demonstrate definite enhancement, making them impossible to distinguish from dysplastic nodules or even HCC in some cases.

2.3. Dysplastic Nodules

Sakamoto et al. and other Japanese investigators have proposed that HCC frequently develops from pre-existing regenerating nodules that have undergone metaplastic or dysplastic change *(5,6)*. In 1995, the International Working Party proposed "Terminology of Nodular Hepatocellular Lesions" *(7)*. Hepatocellular nodules were classified as follows: regenerative nodule, dysplastic nodule–low grade, dysplastic nodule–high grade, small HCC (<2 cm), or HCC (>2 cm). Analogous to a colonic adenoma evolving into a colonic carcinoma, this theory proposes that some overt HCCs are the end

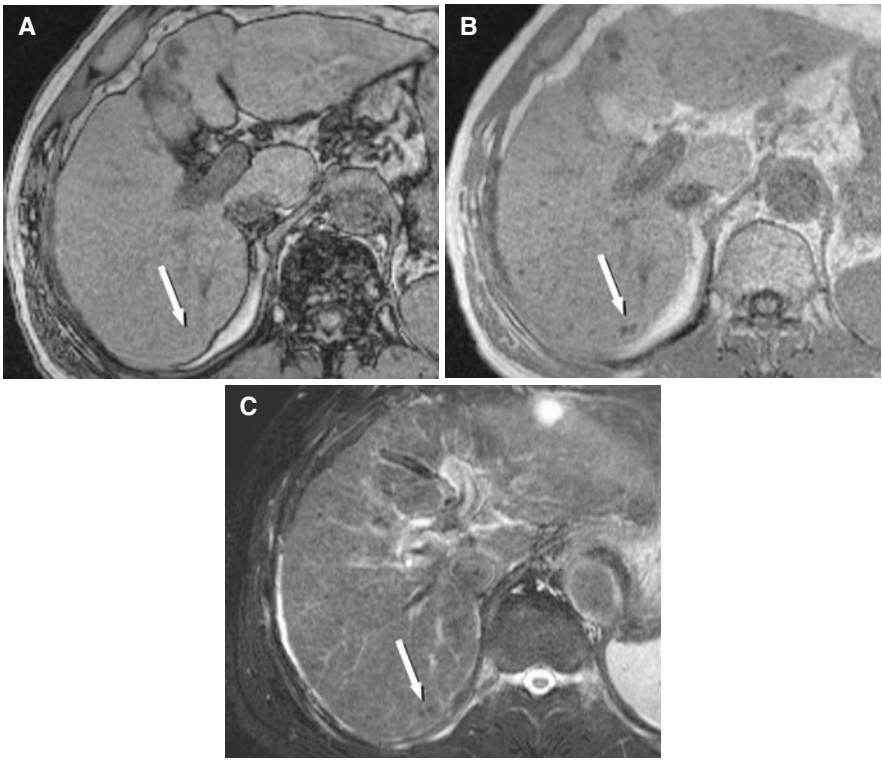

Fig. 3. Regenerating nodules. (**A**) Out-of-phase T1-weighted gradient echo (TE = 2.2 ms) image shows faint low intensity representing siderotic nodules in segment VI. (**B**) In-phase T1-weighted image (4.2 ms) demonstrates darker (hypointense) and blooming subcentimeter lesions in corresponding area. (**C**) T2-weighted image shows the same lesion is also hypointense to liver.

result of a multistep evolution of regenerating nodule to a low-grade than a high-grade dysplastic nodule and subsequently into HCC. Accordingly, dysplastic nodules are considered premalignant. Dysplastic nodules are found in 11–25% of explanted livers at transplantation *(8,9,10)*. In a recent report (11), cumulative HCC development rates at the first, third, and fifth year were 46.2, 61.5, and 80.8% for high-grade dysplastic nodule; 2.6, 30.2, and 36.6% for low-grade dysplastic nodule; 3.3, 9.7, and 12.4% for regenerative nodule, respectively.

Unfortunately, dysplastic nodules are difficult to recognize on imaging and may have features in common with regenerating nodules or HCC. Dysplastic nodules are reported to show homogeneous low echogenicity and, on Doppler sonography, continuous afferent waveform signals that reflect their portal venous supply, rather than pulsatile arterial flow *(12)*. In our practice we have rarely diagnosed or even correctly suggested the presence of

a dysplastic nodule by sonography. Bennett et al. *(13)* detected only 1.6% of dysplastic nodules within cirrhotic livers by sonography compared with thin-section explanted liver pathologic results.

Because dysplastic nodules receive predominantly portal venous flow, they usually do not demonstrate bright enhancement on arterial phase CT or MR. Therefore, marked arterial phase enhancement should suggest HCC rather than dysplastic nodule, although well-differentiated HCCs often show substantial portal venous rather than arterial enhancement *(10,14)*. A diagnosis of dysplastic nodule can be suggested based on a CT finding of a small nodule ($\leq$2 cm) that is non-encapsulated and hypodense to surrounding liver on enhanced CT scan. However, CT is quite limited in diagnosing dysplastic nodules, with reported sensitivity of 10–34% *(8,10)* and poor specificity as well.

MR offers the most promise in diagnosing dysplastic nodules which are reported to demonstrate iso- or hyperintensity on T1-weighted images and hypointensity on T2-weighed images, quite in contrast to typical findings for HCC *(15)* (Fig. 4). Arterial phase bright enhancement should suggest development of a focus of HCC within a dysplastic nodule, so-called "nodule-in-nodule appearance" (Fig. 5). When these hypervascular foci are obscured in the hepatic arterial dominant phase because the whole hepatic nodule is hyperintense on pre-contrast T1-weighted images, superparamagnetic iron oxide (SPIO)-enhanced MRI may allow more accurate diagnosis of HCC *(16)*. In an excellent study comparing MR with explanted livers among transplantation recipients, however, Krinsky et al. were able to detect only 15% of dysplastic nodules on pre-transplant MR studies *(9)*. Moreover, 4 of 59 dysplastic nodules demonstrated arterial phase enhancement and were mistaken for HCC. Finally some non-dysplastic regenerating nodules were

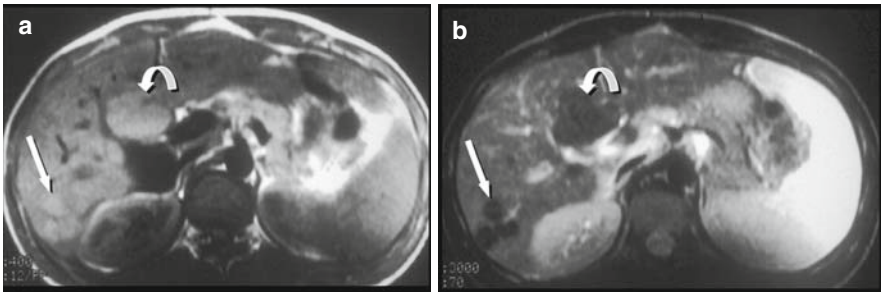

Fig. 4. Dysplastic nodule. (**a**) T1-weighted MR demonstrates 2.0 cm (*arrow*) and 3.0 cm nodules (*curved arrow*) that are slightly hyperintense to surrounding liver. (**b**) T2-weighted MR shows the same lesions are slightly hypointense to liver.

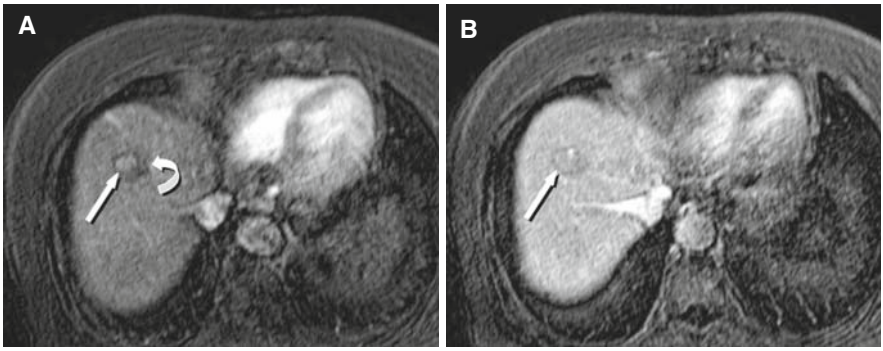

Fig. 5. "Nodule-in-nodule appearance" of HCC. (**A**) Arterial phase MRI shows faint enhancement (*arrow*) of the HCC within the larger hypointense dysplastic nodule (*curved arrow*). (**B**) Portal venous phase MRI shows the internal HCC nodule as iso- to hypointense to liver (*arrow*).

hyperintense on T1 and hypointense on T2-weighted images, further limiting the specificity of MR for this diagnosis.

The typical CT and MR findings that may be helpful in distinguishing among various nodular lesions in the cirrhotic liver are summarized in Table 1.

Table 1
Nodular Lesions in Cirrhosis

	CT				*MR*			
	NC	*HAP*	*PVP*	*Delay*	*T1*	*HAP*	*PVP*	*T2*
Regenerative nodule	— or ↑	—	—	—	— or ↑	—	—	— or ↓
Dysplastic nodule	— or ↑	— or ↑	—	—	— or ↑	— or ↑	—	— or ↓
Well-diff HCC	— or ↓	— or ↓	↓	↓	— or ↑	— or ↑	— or ↑	↑
Mod-diff HCC	— or ↓	— or ↑	— or ↓	↓	— or ↓	↑	— or ↑	↑

— = not seen (isodense, isointense);
↑ = hyperdense (-intense) to liver;
↓ = hypointense (-intense) to liver;
HAP = hepatic arterial phase;
PVP = portal venous phase

3. HEPATOCELLULAR CARCINOMA

Detection of any mass lesion is dependent on its size and the "contrast difference" between the mass and the surrounding liver. Distinguishing a small nodular HCC within the cirrhotic liver is challenging, especially since the "background" liver is usually heterogeneous due to varying amounts of fibrosis, necrosis, fat, regenerating nodules, etc. Almost all imaging tests rely on intravascular administration of contrast media to increase the conspicuity of mass vs. liver, as well as to characterize the hemodynamic features of the mass.

Ultrasonography is often used as a screening modality for high-risk patients and is repeated at frequent intervals. A small HCC may be hypo-, hyper-, or isoechoic on sonography, the latter detectable only if set off by a peripheral halo or pseudocapsule *(12)*. Early work with "microbubble" sonographic contrast agents suggests that they are useful in demonstrating heterogeneous hypervascularity within HCC and may increase the sensitivity and specificity of sonography in diagnosing HCC *(17,18)*. HCC is never diagnosed by sonography alone; percutaneous biopsy, usually preceded by CT, MR, or angiography alone or in combination, is routine. Moreover, even in the small adult, it is difficult to avoid sonographic "blind spots" in the liver due to overlying ribs or bowel gas or excessive fibrosis or fat that attenuates the ultrasound beam.

In most institutions, helical CT has been the mainstay in imaging surveillance of the cirrhotic liver. Multidetector row CT (MDCT) technology and newer MR pulse sequences allow efficient breath-held scanning through the liver prior to contrast administration, as well as during the arterial phase, portal venous phase, and (in special circumstances) delayed or equilibrium phases of the circulating IV bolus of contrast material *(19,20,21)*. It warrants emphasis to state that a CT or MR scan performed without multiple phases of imaging or without the rapid IV bolus administration of contrast medium will miss most small (treatable) HCCs and is nearly useless as a screening test.

CT allows the detection and characterization of most hepatic masses more than 2 cm in diameter. Common benign lesions such as cysts, hemangiomas, and focal fat should be identified with confidence (Fig. 6), and there is ample documentation of the reliability of CT findings in this setting *(22,23)*.

HCCs can have a variety of appearances on CT, but the morphology and hemodynamic characteristics of this tumor are well depicted. Large tumors are heterogeneous, often multifocal, and frequently obstruct or invade intrahepatic bile ducts or the hepatic or portal veins (Fig. 7). Large tumors such as these are relatively easy to detect and stage by CT but are not curable and, as such, represent a failure of screening.

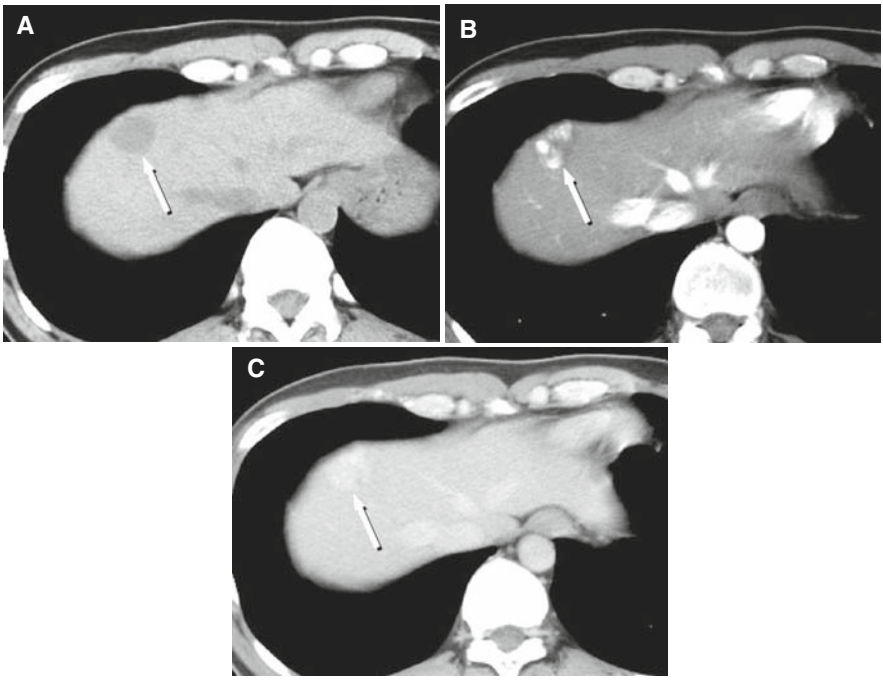

Fig. 6. Small cavernous hemangioma. (**A**) Unenhanced CT. (**B**) Arterial phase-enhanced CT. (**C**) Portal venous phase CT. A 1 cm nodule (*arrow*) in the lateral segment is isodense with blood vessels on all three phases identifying it as an hemangioma rather than HCC.

Aggressive screening should result in detection of much smaller HCCs that are often amenable to treatment, whether for palliation or cure. Small well-differentiated HCCs may still receive predominantly portal venous flow and, therefore, appear relatively hypo- to isodense to liver on the nonenhanced and arterial phase images, and distinctly hypodense to liver on portal venous and delayed phase images *(10,14,24)* (Fig. 8). Most HCCs, even when small, develop increased arterial flow through tumor vessels and are best detected on the arterial phase CT images as a homogeneous or slightly heterogeneous hyperdense mass with rapid washout of contrast resulting in a slightly hypodense mass on portal venous or delayed images (Fig. 9). The delayed or equilibrium phase of imaging can be helpful as an added sequence; some HCC will have a capsule or small foci of fat while regenerating and dysplastic nodules do not.

Caution is necessary to avoid mistaking certain perfusion abnormalities of the liver for hypervascular tumor. A small peripheral wedge-shaped area of increased density seen only on the arterial phase of imaging is a transient

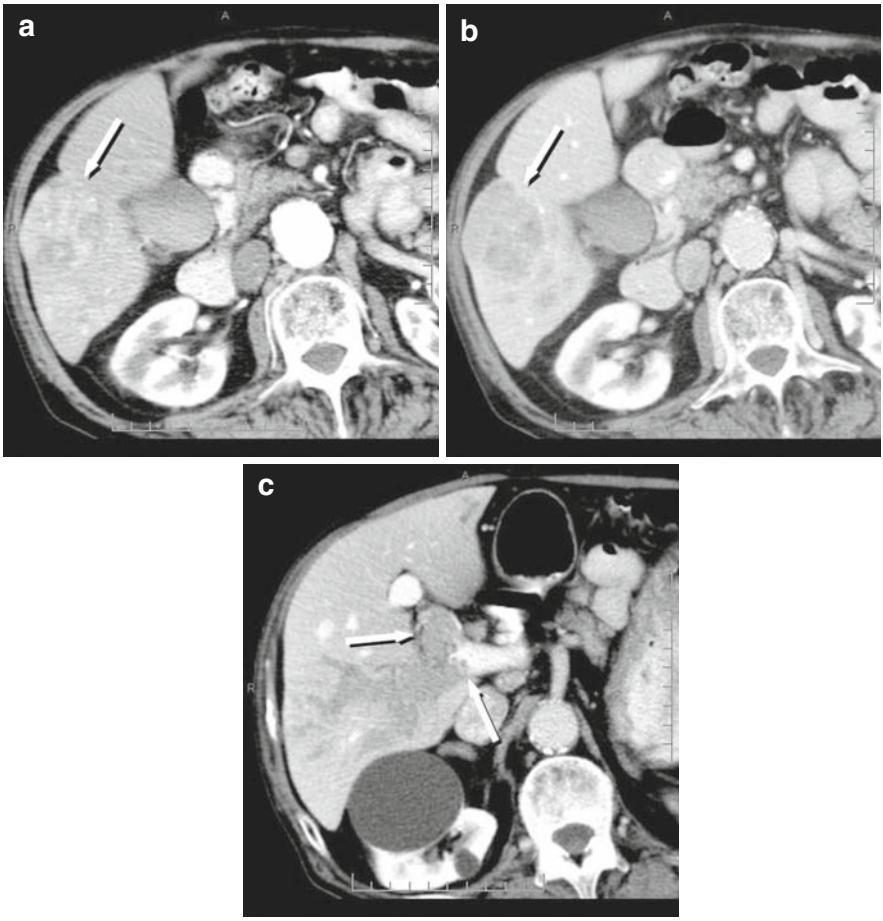

Fig. 7. Hepatocellular carcinoma (HCC). (**a**) Arterial phase CT shows a hypervascular 3 cm tumor (*arrow*). (**b**) Portal venous phase CT shows the HCC as hypodense to liver (*arrow*). (**c**) Portal venous phase CT. The anterior and posterior branches of the right portal vein are occluded by tumor (*arrows*).

hepatic attenuation difference (THAD) and is usually due to arterioportal shunts or aberrant venous drainage *(25,26)*. Other researchers have described several kinds of non-neoplastic lesions that are seen as early-enhancing foci during the hepatic arterial phase, potentially mimicking hypervascular neoplasms; the causes include non-neoplastic arterioportal shunting *(27,28)*, portal vein obstruction *(29)*, cystic venous drainage *(30)*, or compression effect *(31)*. Larger segmental or even lobar enhancement differences should prompt close scrutiny for portal venous occlusion or invasion which may result from HCC.

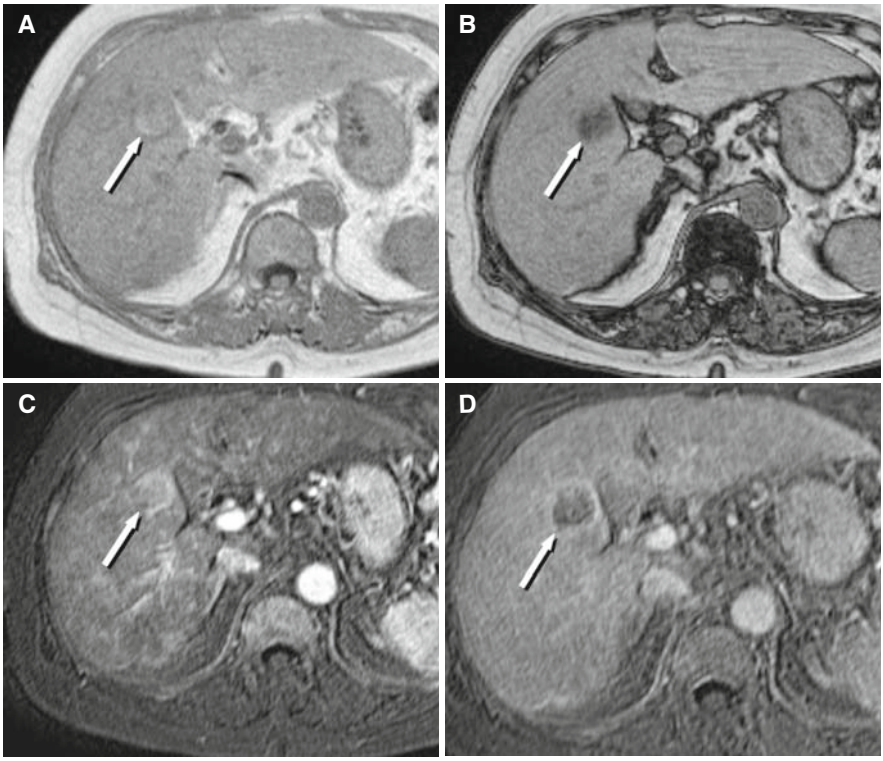

Fig. 8. (**A**) Fat-containing well-differentiated HCC. (**B**) Out-of-phase T1-weighted image. The mass (*arrow*) shows marked hypointensity indicating signal suppression due to lipid content of the HCC. (**C**) Gadolinium-enhanced hepatic arterial phase image. The mass is slightly enhanced (*arrow*). (**D**) Delayed phase image shows the mass as hypointense to liver (*arrow*).

Well-differentiated HCC often contains microscopic or macroscopic deposits of fat which imparts characteristic imaging features. Intralesional fat renders the HCC hyperechoic on sonography, hypodense on noncontrast CT, and hyperintense on T1-weighted MR (Fig. 8). Some HCCs are surrounded by a complete or partial "capsule" that may be fibrotic and visible as hypodense on nonenhanced CT (and T1-weighted MR) but become hyperdense on delayed enhanced CT (or T2-weighted) images.

HCC can be variably intense on T1-weighetd MR (35% hyper-, 25% iso-, 40% hypointense), but almost all are hyperintense on T2-weighted images *(32)*. Multiphasic imaging following bolus administration of IV contrast medium is just as essential for MR evaluation of HCC as for CT. The usual intravenous agent is gadolinium (Gd-DTPA, gadopentetate dimeglumine). Arterial, portal venous, and delayed phase imaging demonstrate

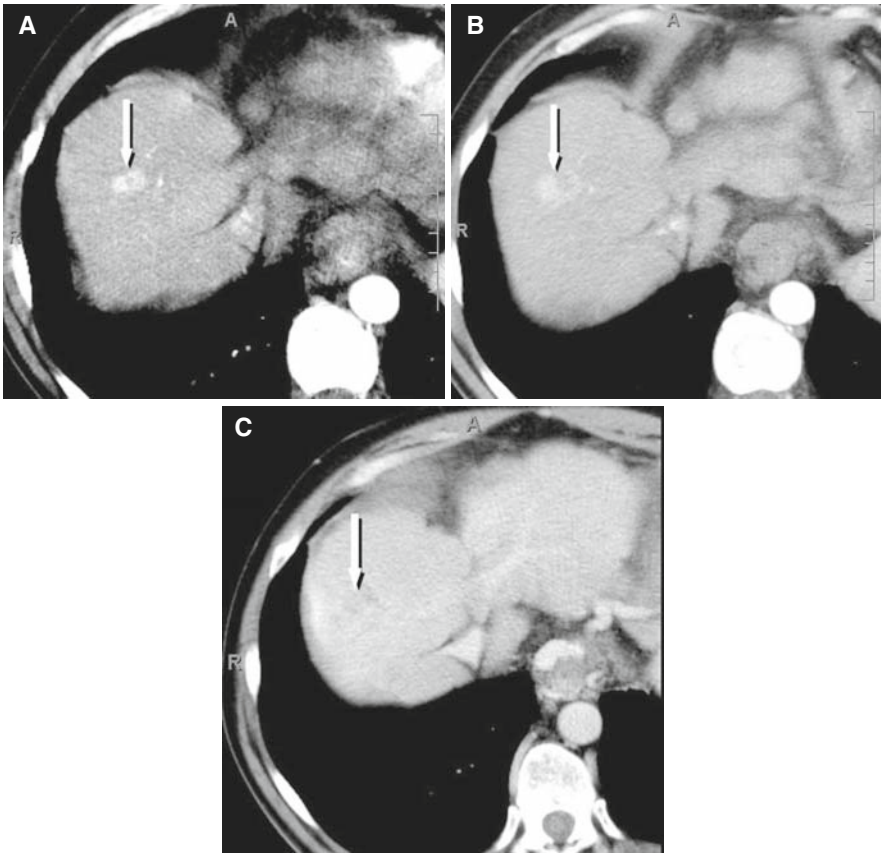

Fig. 9. Surveillance for HCC. (**A**) Arterial phase CT shows tiny enhancing lesion (*arrow*) which is difficult to distinguish between small HCC and arterioportal shunting. (**B**) Arterial phase CT obtained after 5 months. The nodule (*arrow*) has increased in size. (**C**) Portal venous phase CT. The HCC (*arrow*) is now slightly hypodense to liver.

the same hemodynamic tumor characteristics as detailed for CT *(20,32)*. Recently, Kim *(33)* has compared the diagnostic performance of gadobenate dimeglumine-enhanced MRI with 16-MDCT for the detection of HCC. They reported that gadobenate dimeglumine-enhanced MRI had a higher sensitivity for small HCCs, although the false-positive rate was higher due to the nonspecific enhancement of benign lesions (such as an arterioportal shunt). While debates continue between CT and MR proponents, the two modalities appear to have comparable diagnostic accuracy. However, in some clinical settings, MR imaging may be preferred over CT given the reduced patient exposure to ionizing radiation and the ability to use a non-iodinated contrast medium.

Liver-specific MR contrast agents are occasionally useful in evaluation of masses within the cirrhotic liver. One class of these agents, the superparamagnetic iron oxides (ferumoxides), is phagocytized by Kupffer cells and accentuates the difference between normal liver and tissue that lacks Kupffer cells. Another class of agents, including mangafodipir (Teslascan, Amersham, Princeton, NJ), is incorporated into functioning hepatocytes and is useful in detecting nonhepatocellular masses. Unfortunately, well-differentiated HCC often contains Kupffer cells and functioning hepatocytes and may not be detected as a tumor *(34)*. Moreover, in the cirrhotic liver scarring and inflammation may result in decreased uptake of the contrast agents. These agents might help to evaluate the histological grade of HCC, but the practical value of this is uncertain.

4. ACCURACY OF SONOGRAPHY, CT, AND MR AS SCREENING MODALITIES

Many reports claim accuracy, sensitivity, and specificity of over 90% for CT and MR in diagnosis of HCC, and only slightly less for sonography. Most of these are retrospective studies, report predominantly on large tumors that were known or suspected prior to imaging, lack a gold standard of proof, and suffer from numerous sources of bias. The most reliable reports are based on investigations comparing the imaging test with pathological exam of the explanted liver or with a combination of sophisticated imaging tests, resection, biopsy, and clinical follow-up. We will focus on several studies that meet these criteria.

Bennett et al. *(13)* correlated pre-transplant sonography results with explant pathology in 200 patients. Ultrasound detected tumors in only 30% of patients; individual lesion detection sensitivity was 21%. We have had similarly poor success with ultrasound screening in Pittsburgh *(35)*.

Our team in Pittsburgh *(36)* studied 195 patients who had transplantation following single-slice helical dual-phase CT, and 32 patients (16%) were found to have HCC in the explanted liver. We were able to detect these by CT prospectively in only 19 of 32 patients (59%) and found only 23 of 63 HCCs (36%). Eighty-two percent of the HCCs in our series were less than 20 mm in size. Tumor detection rates were higher with CT performed within 60 days before transplantation; some tumors surely arose or grew in the longer intervals between scanning and transplantation.

Lim et al. *(10)* studied 41 patients who had multiphase CT prior to liver transplantation; 15 of these patients had 21 HCC nodules found in the explanted liver with a mean diameter of 19 mm. These investigators were able to detect HCC in 80% of patients (12 of 15) and they identified 15 of 21 HCC (71% sensitivity).

Murakami et al. *(26)* studied 51 patients with 96 hypervascular HCCs, using the latest generation of MDCT (or "multislice" CT) and multiphasic imaging that included two sets of arterial phase images. Double arterial phase imaging showed significantly greater sensitivity and specificity than either phase alone, with an overall sensitivity of 86% and positive predictive value of 92%. The double arterial phase imaging also allowed them to avoid some false-positive diagnoses due to arterioportal shunts. The mean size of HCCs in their series was 22 mm and almost half the lesions were less than 2 cm in diameter. Hypervascular HCCs are clearly imaged best during the phase of maximum tumor enhancement and minimal hepatic parenchymal enhancement, and this arterial phase may last only a few seconds. Owing to variations in tumor vascularity and patient cardiovascular status, some means of optimally timing the bolus of contrast and initiation of imaging is essential.

Krinsky et al. *(9)* performed multiphasic MR in 71 patients who had transplantation and pathological correlation of the explanted liver with the prospective MR interpretation. MR enabled diagnosis of HCC in only 6 of 11 patients (54%) who had HCC and only 10 of 19 tumors (53%). The mean size of the HCCs that were missed was 13 mm. Four patients each with confluent hepatic fibrosis and dysplastic nodules had a false-positive diagnosis of HCC.

Excluded from the Krinsky study and our Pittsburgh report were patients who had HCC known or suspected prior to MR or transplantation. Reporting exclusively on patients with HCC who have had transplantation probably underestimates the accuracy of CT and MR for several reasons, including the close scrutiny for small lesions in the explanted liver that may not have otherwise come to clinical attention. In addition, many patients are excluded from transplantation because CT or MR demonstrates advanced HCC, removing them from the study population. Higher sensitivity and specificity can be achieved in patient populations that include larger tumors or those which are symptomatic or associated with markedly elevated serum tumor markers.

5. WHY, WHEN, AND HOW TO SCREEN

It is clear that detection of curable or treatable HCC by imaging is challenging but newer therapeutic options make this a worthwhile goal. Small HCCs are amenable to resection or various ablation techniques, such as alcohol injection or radio-frequency coagulation, and surgical treatment for smaller tumors has resulted in improved 5-year survival *(37)* (Fig. 10). Liver transplantation is an appropriate option for patients with small tumors, with reports of recurrence-free survival rate of 85% following transplantation in patients with early-stage HCC (one lesion <5 cm or up to three lesions $\leq$3 cm) *(38,39)*.

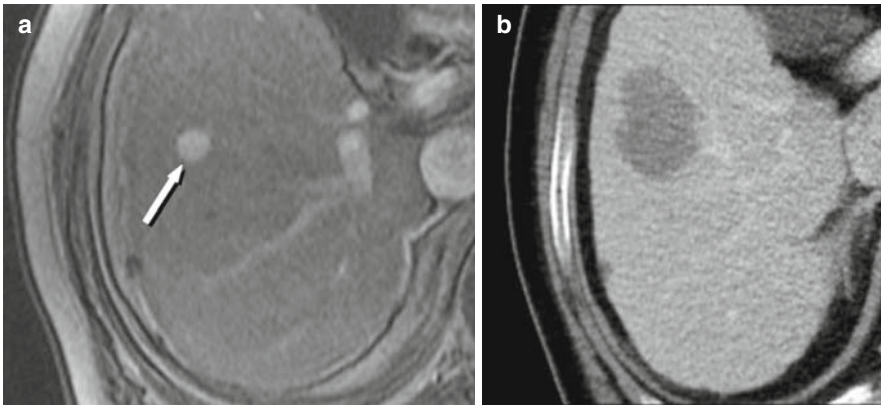

Fig. 10. Small HCC treated with radio-frequency ablation. (**a**) Arterial phase MR, T1-weighted image shows a 1 cm hypervascular nodule (*arrow*). (**b**) Following percutaneous RF ablation under ultrasound guidance the ablation defect is shown, with no viable tumor on enhanced CT.

The European Association for the Study of the Liver (EASL) convened a panel of experts on HCC in Barcelona in September 2000 and has published their findings and recommendations for surveillance and management of HCC *(40)*. They note that the prevalence and etiology of HCC vary markedly throughout the world but the most significant risk factor is the presence of cirrhosis, regardless of its etiology. Once cirrhosis is established the main predictors of HCC are male gender and increased levels of α fetoprotein (AFP). However, AFP is not a very good screening test since it has a sensitivity of 39–64%, a specificity of 76–91%, and a positive predictive value of 19–32% *(41,42)*.

The Barcelona panel recommended ultrasonography as the preferred surveillance tool but noted that sonography is highly operator dependent and requires specific training and interest to acquire the skills necessary to detect early HCC. The European group has recommended that sonography be repeated every 6 months along with serum AFP levels. If the AFP becomes elevated or if a liver nodule is detected by sonography, they recommend helical CT (or MRI or angiography) for further evaluation.

Recommended intervals between surveillance tests are based, in part, on estimates of tumor growth rate. The doubling time of HCC lesions less than 2 cm has been estimated at 2–12 months *(43–45)*. The Barcelona panel has set a goal of detecting tumors below 3 cm in diameter and recommends surveillance at 6-month intervals, while some Japanese groups are much more aggressive, recommending serum AFP and/or PIVKA measurements every 2 months, sonography every 3 months, and CT or MRI every 6 months *(24)*. This surveillance protocol is applied to patients with established cirrho-

sis; for patients with chronic hepatitis without established cirrhosis, the intervals are doubled (e.g., AFP every 4 months, sonography every 6 months, CT every 12 months). Murakami et al. *(24)* report that this screening protocol has resulted in 20–30% of the HCC nodules being detected in Japan while less than 2 cm in diameter and 50–60% at less than 5 cm.

We believe that some modification of these screening protocols may be necessary for applicability to a North American setting for several reasons. In spite of recent increases in the prevalence of chronic hepatitis in this country, the prevalence of HCC is still much lower than in Asia or Southern Europe making the disease and its manifestations less familiar to American physicians. For a surveillance program to work properly, patients must be evaluated in their own community; referral to specialized centers usually occurs only after a disease process is documented and treatment is initiated. In most North American medical settings hepatic sonography will be an ineffective screening tool, in part because American physicians are not likely to perform the detailed dedicated sonographic analysis of the cirrhotic liver necessary to detect and distinguish focal hepatic masses. American cirrhotic patients are also more likely to be larger and to have hepatic steatosis, factors which further limit the accuracy of sonography.

MR imaging is less appealing as a routine screening test because it is less widely available, more expensive, and less acceptable to many patients. There are considerable technical differences between individual MR scanners, making it difficult to apply specific imaging protocols or to obtain reproducible results from one setting to another. Nevertheless, MR may be the single most accurate imaging test assuming optimized technique and expert interpretation.

Helical CT is likely to remain the predominant imaging modality for detection and staging of HCC in North America. Technical improvements, especially the rapid emergence of multidetector row (multislice) CT, have resulted in improved accuracy that rivals that of more expensive and invasive studies such as CT catheter angiography and portography. The frequency with which CT should be employed for surveillance is likely to remain controversial. We believe that the Barcelona recommendations are too restrictive in the use of CT. It is noteworthy that many Japanese investigators employ CT and more invasive studies very liberally in spite of their enthusiasm for ultrasonography. Ultimately, the choice and timing of screening tests will depend on many factors including the etiology and stage of chronic liver disease, level of serum tumor markers, and local expertise and availability of high-quality imaging. The rapid development of innovative contrast media and improved ultrasound, CT, and MR scanners makes it mandatory for all physicians involved in the care of patients with chronic liver disease to stay abreast of new developments and to implement these into their own practices.

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13 MRI for Detection and Evaluation of Hepatocellular Carcinoma

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CONTENTS

ABSTRACT

MRI is a useful method of imaging the cirrhotic liver, including for detection and evaluation of hepatocellular carcinoma (HCC), both for its initial diagnosis and following its response to management. In this chapter, we discuss features which allow distinction of HCC from other lesions in the cirrhotic liver, such as regenerative nodules, confluent fibrosis, and benign enhancing pseudonodules. One major strength of MRI is its use of multiple pulse sequences, analogous to the use of various stains for histopathology. Pulse sequences with unique value for characterizing focal liver lesions include T1-weighted, T2-weighted, lipid-sensitive, and multiphasic contrast-enhanced images. Features that facilitate diagnosis of HCC include its shape, capsule, internal nodularity, signal intensity, and sequential

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pattern of dynamic contrast enhancement. It is particularly important that radiologists and clinicians reach understanding on terminology for expressing confidence that a given focal lesion is HCC or benign, so that reported findings are most useful for guiding management decisions. A suggested framework for categorizing this confidence is provided.

Key Words: MRI; liver; hepatocellular carcinoma

Evaluation and management of patients with cirrhosis present many challenges, one of which is the reliable detection of hepatocellular carcinoma (HCC) at a stage when treatment can improve the length and quality of a patient's life. As with other cancers, the potential value of imaging for initial detection depends on many factors, which are the following:

1. Is there a population of high-risk individuals who can be identified for screening by imaging?
2. Is imaging capable of detecting the malignancy earlier than clinical or laboratory methods?
3. Is there an effective method for treating the malignancy at the stage when it is most likely to be detected?
4. Do the benefits of early detection and treatment compare favorably with the financial and other costs of the imaging screening program?

In the case of HCC, the answers to all of the above questions are yes. Patients with cirrhosis, especially of viral etiology, are at high risk for developing HCC *(1–4)*. Magnetic resonance imaging (MRI), computed tomography (CT), and ultrasound can all detect HCC, often before α-fetoprotein and other nonimaging signs allow its diagnosis *(5–9)*. Imaging can be used judiciously to diagnose small HCC, obviating biopsy when imaging diagnostic signs are particularly compelling *(10)*. HCC can be locally treated by many methods, often improved when used in combinations, including chemoembolization, radioembolization, chemical ablations (e.g., ethanol or acetic acid), and RF ablation *(4*, *9*, *11–17)*. The success of these methods might possibly be further augmented when combined with systemic therapy, such as with agents that target VEGF receptors and tumor-induced angiogenesis *(18–20)*. If the local and systemic treatments mentioned above can prevent or prolong the interval before HCC spreads to extrahepatic sites, liver transplant can be used to cure the patient of HCC and prevent recurrence *(11*, *21–25)*. Therefore, HCC presents itself as a particularly valuable opportunity for imaging to improve the lives of patients at risk for this malignancy *(2)*.

In this chapter, I will make some general comments about the challenges that must be addressed to detect HCC within a cirrhotic liver. I will provide a

framework to reduce some confusion regarding terminology that may appear in the imaging literature and in clinical imaging reports. I will then discuss several of the features that help distinguish HCC from other focal findings in cirrhotic livers. Finally, based upon limited literature and some perspective gained from clinical experience, I will offer some suggestions about how the use of MRI for detecting hepatocellular cancer may proceed during the next few years.

1. FOCAL IMAGING FINDINGS IN CIRRHOTIC LIVER

As cirrhosis develops and progresses, the remaining liver parenchyma consists of regenerative nodules of variable size, surrounded by fibrous septations. The first step in evaluating images of a cirrhotic liver is the recognition that the tissue between the fibrotic septations, that is, the regenerative nodules, should generally resemble healthy hepatocellular parenchyma. The abnormal appearance of a cirrhotic liver is caused by alterations in shape due to the combination of scarring, atrophy of some portions, and hypertrophy of others, as well as abnormal signal imparted by the presence of fibrosis and inflammation.

Once a focal part of the liver is noted that appears different compared with the surrounding liver, the next task is to determine whether this tissue is more or less abnormal than the remaining liver parenchyma *(26)*. For example, a relatively sparred area within a severely diseased liver can resemble a mass, when in fact the focal finding is less diseased than the surrounding tissue. The challenge here, before even considering whether there is evidence of malignancy, is to categorize the following benign tissues:

1. *Regenerative nodules*. In fact, the entire cirrhotic liver consists of regenerative nodules. Therefore, any distinct nodule that looks different from the background liver should arouse at least a modest level of suspicion.
2. Confluent fibrosis and severely damaged liver, containing few if any hepatocytes, will look distinctly different from healthy liver parenchyma. Confluent fibrosis is darker on T1-weighted images and brighter on T2-weighted images, features shared by most malignancies, including some HCCs. Confluent fibrosis is therefore best distinguished from HCC by its shape, which is geographic rather round, and by retraction of liver shape, rather than expansion *(27)*.
3. Benign enhancing pseudonodules are the most common problem leading to false-positive diagnosis and frequent follow-up imaging examinations. As discussed toward the end of this chapter, subcentimeter

foci of transient enhancement are extremely common in a cirrhotic liver and are usually benign *(28–32)*. Frequent short-term follow-up of these common benign findings therefore threatens to dramatically increase the overall cost of an imaging screening program, and should be minimized to whatever extent possible *(33)*.

4. Hyperplastic nodule has only scant description in the literature *(34–39)*, although it is probably a common cause of false-positive MRI. Like the more common regenerative nodule, a hyperplastic nodule is composed entirely of benign liver tissue. In fact, there is often minimal distinction between these two entities in the pathologic literature, due to their absence of dysplastic or neoplastic cellular features *(40)*. The main distinction between these two nodular entities is their blood supply, which causes dramatic differences on contrast-enhanced imaging studies but may have little or no effect on their light microscopic appearance. Hyperplastic nodules are thought to arise as a response to alterations in portal venous perfusion, giving rise to nodular hypertrophic tissue with vascular supply entirely from hepatic arteries, without meaningful contribution from portal veins *(41)*. Hyperplastic nodules are most common in the setting of Budd-Chiari syndrome but can arise in any scenario where portal venous perfusion is abnormal, including in patients with cirrhosis. In the setting of an otherwise normal liver, these nodules are termed focal nodular hyperplasia (FNH). In fact, in patients with Budd-Chiari syndrome or cirrhosis, the term "FNH-like nodule" has been used *(35–37)*. This is an unnecessarily redundant term, so the more generic and simpler term hyperplastic nodule is preferable. Hyperplastic nodules are considered entirely benign, without premalignant nature, and should not be confused with dysplastic nodule.
5. Dysplastic nodule is a borderline lesion, with atypical cellular features different from those of regenerative or hyperplastic nodules but not meeting criteria for overt malignancy *(40*, *42*, *43)*. They are considered premalignant, and foci of HCC may develop within them. Dysplastic nodules can be visible on imaging studies, although their features overlap those of some regenerative nodules and some HCCs. Therefore, dysplastic nodule can be included in the differential diagnosis of a nodule in a cirrhotic liver, but at this point, dysplastic nodule is not a specific diagnosis that can be offered by imaging.

HCC is the subject of this entire book and need not be defined here. Rather, in the next section I will describe imaging features of HCC and indicate how these may be used to distinguish them from other nodules and focal findings in the cirrhotic liver.

2. MRI: PULSE SEQUENCES AND GENERAL CONSIDERATIONS

Like other methods of imaging, MRI can depict hepatic and abdominal anatomy. However, MRI also offers a more robust and comprehensive set of tools for characterizing tissue. It is therefore customary for most MRI exams, particularly hepatic MRI, to include multiple pulse sequences repetitively interrogating the same tissue, often in identical image planes. In this respect it is analogous to light microscopy, where the same histologic structure is repeatedly evaluated using different stains, each designed to highlight a particular tissue component of interest. Most MRI examinations will include the following.

2.1. Survey Images

These typically include coronal images but may also include sagittal and transverse images. They provide a brief survey of the abdomen in 1 minute or less and help determine the region of the abdomen to be included in the remainder of the examinations. On occasion, the position of the patient and local receiver coils may need to be changed to best optimize the signals received.

2.2. T1-Weighted Images with Lipid and Iron Sensitivity

T1 is a characteristic of tissues, whereby short T1 leads to high signal intensity (bright on the images) on T1-weighted images. In order of increasing T1 (decreasing brightness on T1-weighted images) are adipose tissue, liver parenchyma, most other tissues including malignancies, and simple cysts. On basic T1-weighted images, adipose tissue is therefore bright, liver medium, and simple cysts dark. HCC has variable appearance and therefore can be dark, intermediate, or bright on T1-weighted images *(44*, *45)*.

Inherent differences between the protons in water and the protons in lipid can be exploited, in various ways, to separate the signals from water vs. lipid protons. It is now routine to obtain T1-weighted images as a pair of images, based on two consecutive echoes *(46–48)*. One of these is "in-phase," where the signals of water and lipid protons add together. The other is "opposed-phase," whereby water protons and most protons from lipid interfere destructively, so that points in the image that contain water and fat, such as fatty liver parenchyma, show up as darker compared with in-phase images. These two paired images, obtained at exactly the same time and place, can either be visually compared or be postprocessed to generate difference images. It is also standard, at some point in the examination, to obtain T1-weighted images where lipid protons are selectively suppressed, generating "fat-suppressed T1-weighted images."

2.3. T2-Weighted Images

These images accentuate differences in the T2 between different tissues. Like T1, T2 is characteristic of tissues. T1 and T2 commonly, but not always, parallel each other. For example, both simple cysts and cerebral spinal fluid have extremely long T1 and long T2, and are therefore bright on T2-weighted images. Liver is dark on most T2-weighted images, whereas moderately to poorly differentiated HCC is usually brighter on these images, similar to spleen. Images can be made more T2 weighted by lengthening the echo time (TE). It is common to obtain two different sets of T2-weighted images, one with moderate T2 weighting to show liver tumors and enlarged lymph nodes, and one with heavy T2 weighting to show fluid as much brighter than solid tissue. In fact, extremely heavily T2-weighted images are commonly obtained to accentuate biliary and pancreatic ducts to form magnetic resonance cholangiopancreatography (MRCP) images. Heavily T2-weighted images are helpful for distinguishing benign cysts and hemangiomas from solid tissue, including HCCs.

2.4. Dynamic Multiphasic Contrast-Enhanced Images

These images are routine and considered essential for sensitive detection of HCC. As a minimum, four separate sets of T1-weighted images, usually with fat suppressed 3D thin-slice technique, are obtained. These included unenhanced images, images obtained during the first pass of contrast material through arteries (arterial phase), images obtained about 20 seconds after the arterial phase (blood pool or venous phase), and images obtained three or more minutes after contrast material has been allowed to equilibrate throughout the vascular and interstitial spaces (delayed or extracellular phase images).

Most HCCs will be bright on arterial phase images due to their predominant supply by arterial rather than portal venous perfusion, and most will be less intense blood pool or delayed phase images (probably because of less fibrosis in HCC compared with background liver parenchyma).

There is a new class of gadolinium contrast agent that has partial hepatobiliary excretion, including gadobenate dimeglumine and gadoxetic acid disodium *(49–51)*. These agents have weak binding to serum proteins, approximately doubling their effect on MR images at a given dose. An additional advantage of these agents is increased enhancement of liver tissue compared with most tumors during delayed phase imaging, after contrast agent has been primarily cleared from blood.

There are some additional images that are included in some protocols because of their potential to provide additive value or confirmation of information from other sequences, but are not necessarily routine.

Diffusion weighted images utilize microscopic water motion to highlight differences between tissues *(52–57)*. Generally, malignant tumors have

restricted water motion compared with many benign tissues. *MR spectroscopy* allows detailed analysis of chemical differences depending on molecular structure, either of protons or other nuclei, but usually with much lower spatial resolution *(58)*. At the present time, neither of these techniques should be considered routine or essential for detecting HCC.

Bright-blood images can be used to demonstrate blood vessels, using either motion-compensated techniques to show the water in blood or use the motion of the blood to show patent vessels. These images are often included if the contrast-enhanced images are technically inadequate due to motion or other artifacts, or if gadolinium contrast agent is not given.

Perfusion imaging. Advances in MRI hardware and pulse sequence design as well as image postprocessing can extend the value of dynamic contrast enhancement so that images are repeated at more rapid intervals. As a first step, early and late arterial phase images can be obtained during one breath hold. Further increases in speed are also possible, and signal intensities at various phases can be measured and applied to various perfusion algorithms to further characterize tissue. The broad class of perfusion imaging has been used to characterize properties of angiogenesis. It is possible that this method of image analysis may prove useful for characterizing response to new treatments such as VEGF antagonists *(18*, *57*, *59–62)*.

Particulate contrast agents. This class of contrast agent, usually consisting of iron oxide particles that accumulate avidly in Kupffer cells and other cells of the reticuloendothelial system, can darken the surrounding liver and thereby improve the visibility of HCC on appropriate MR images *(11*, *21*, *24*, *25*, *63–65)*. The most successful use of iron oxide contrast agents is in "double contrast MRI" when combined with gadolinium contrast agents *(66–71)*. The increased cost of using two contrast agents has prevented adoption of this technique at most centers.

3. CHARACTERISTICS OF HCC

On *T1-weighted MR images*, HCCs can be dark, intermediate, or bright relative to background liver parenchyma. In spite of this extreme variability, T1-weighted images are still useful. For example, hemangiomas, cysts, and most other malignancies are more consistently dark on T1-weighted images, so intermediate or high signal helps to exclude these alternative diagnoses. Comparison of in-phase and opposed-phase images allows detection of even small quantities of lipid, a common finding in HCCs but not present in liver masses that are not derived from hepatocytes, such as hemangiomas, metastases, or cholangiocarcinoma. High signal intensity on both in-phase and opposed-phase images indicates hepatocellular tissue with copper *(72–74)*. These nodules may be HCC, dysplastic nodule, or other liver tissues with cholestasis.

T2-weighted images are often useful for depicting malignant liver tumors as brighter than background liver, although many HCCs have low signal intensity or be invisible on T2-weighted images *(75)*. The main value of T2-weighted images for evaluating suspected HCC is their specificity. A solid round mass in a cirrhotic liver with high signal intensity on T2-weighted images is usually HCC.

The *shape* of a focal liver abnormality is quite helpful. HCCs are usually round, ovoid, or lobulated. HCCs can produce geographic abnormalities after they invade portal veins and disseminate by a portal venous spread.

A *capsule or pseudocapsule* is a common finding surrounding hepatocellular cancer. A capsule appearance is generally not seen with other focal liver lesions such as dysplastic nodule, hydroplastic nodule, or adenoma.

Internal nodularity (mosaic appearance) is a characteristic of HCC caused by variable dedifferentiation of foci within a dysplastic or a neoplastic mass *(76)*. Benign entities such as dysplastic or hyperplastic nodule or liver regeneration have simpler texture, without internal nodularity. At an early stage, a "nodule-in-nodule" configuration results from focal dedifferentiation to HCC within a dysplastic nodule *(42, 77–79)*. A similar appearance can result from focal further dedifferentiation into less differentiated carcinoma within a well-differentiated carcinoma. Therefore, one or more nodules within a focal mass are a strong characteristic of HCC. These focal dedifferentiated nodules will usually have higher signal on T2-weighted images, lower signal on T1-weighted images, and more arterial vascularity. They will also tend to have rounder shape, as they exert mass effect on the less rapidly growing more differentiated remainder of the tumor.

The *dynamic contrast-enhanced series* is the single most important part of an MRI examination for HCC. The characteristics of the dynamic contrast MRI series are in many respects mimicked by dynamic multiphasic CT. One important advantage of MRI over CT is the complimentary value afforded by the additional MRI pulse sequences, which do not have analogous CT counterparts. Additionally, MRI spares the patient repeated exposures to ionizing radiation and iodinated contrast material.

The arterial phase images are the single most sensitive series for detecting HCC. However, there are caveats. While this series may be more sensitive than any single other series, there are indeed HCCs that may be visible only on other pulse sequences, not on arterial phase images *(31, 80)*. Additionally, benign nodules are often seen as hyperintense on arterial phase MR images. In fact, more than 90% of small nodules seen only on arterial phase images are benign *(29, 30, 81)*. The specificity of dynamic contrast-enhanced series is improved greatly if the nodule is visible on at least one additional series. Most commonly, this will be a "washout appearance," whereby a nodule that is hyperintense on arterial phase images is hypointense on blood pool (portal venous) phase or delayed phase images. Additionally, a nodule that is visible on an unenhanced image and then shows increased enhancement relative to

liver during the arterial phase is more likely to be malignant than a nodule that is visible only on arterial phase images *(31)*.

Three illustrative cases are provided in Figs. 1, 2 and 3.

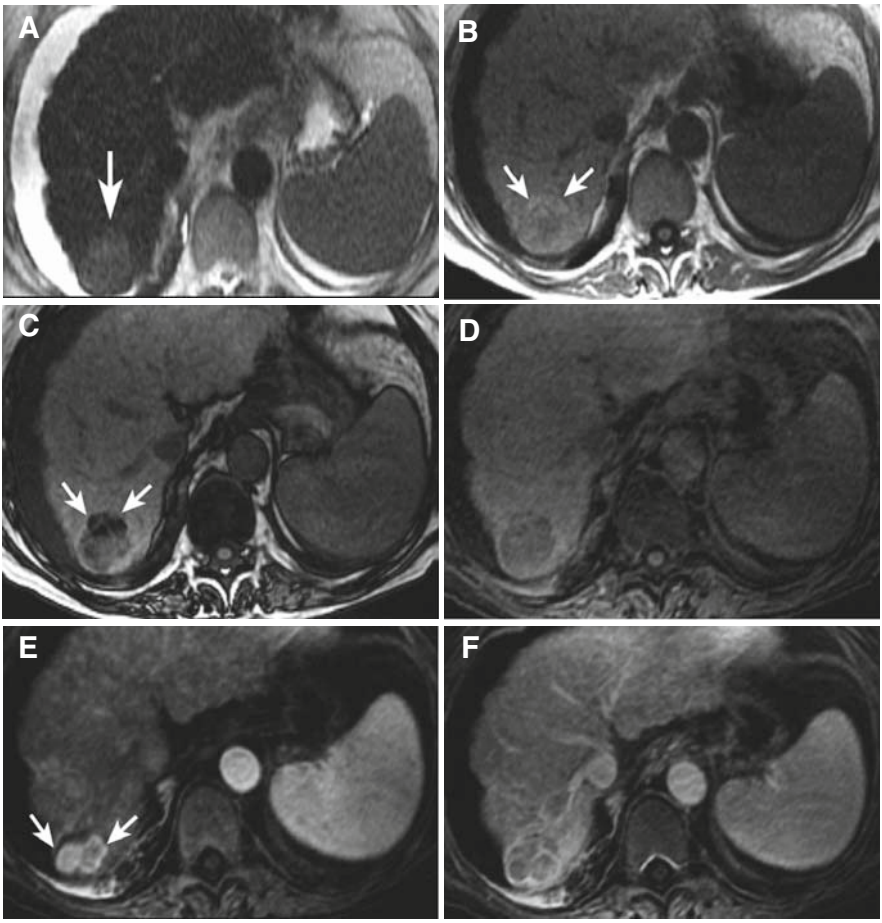

Fig. 1. HCC with many typical MRI features. **A**. T2-weighted image shows HCC as high signal intensity (*arrow*). **B**. T1-weighted image in-phase (water plus fat) shows that most of HCC has similar intensity to remainder of liver, other than increased signal of anterior crescentic portion (*arrows*). **C**. T1-weighted image opposed-phase (water–fat cancellation) shows that HCC lost signal relative to other tissues, indicating lipid content. The anterior crescentic portion (*arrows*) has highest fat content and has therefore lost the most signal. **D**. T1-weighted fat-suppressed image shows the HCC as less signal than the remainder of liver. **E**. As in D, immediately after intravenous injection of gadolinium contrast agent. Hypervascular nodules within the HCC show strong enhancement (*arrows*). **F**. As in E, about 1 minute later. The HCC is now less intense than liver, with a multinodular appearance.

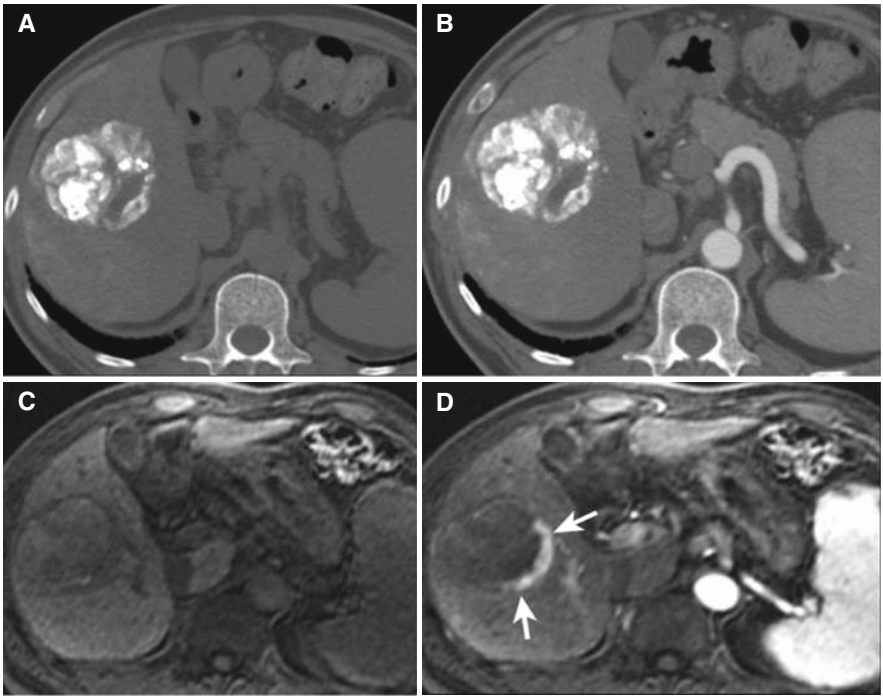

Fig. 2. HCC following chemoembolization, with small remaining viable portion. **A**. Unenhanced CT shows embolic material within HCC. **B**. Contrast-enhanced CT does not show any visible enhancement of tumor. **C**. Unenhanced MRI shows that HCC is of similar intensity to liver. **D**. Arterial phase MRI shows viable hypervascular tissue at the periphery of HCC (*arrows*).

4. REPORTING FINDINGS SUSPICIOUS FOR HCC

The success of screening for HCC depends on detecting the tumor while it can still be treated, without resulting in a frequency of false-negative results that could undermine funding or compliance. Thus far, there is no sufficient data to determine whether repeated imaging at 6-month intervals is superior to annual imaging *(82, 83)*. Our approach has been to attempt confident noninvasive diagnosis with high accuracy while minimizing the frequency of "overdiagnosis" of benign enhancing lesions as HCC. A recent study at our center confirmed that small HCCs that were initially diagnosed as probably benign did not progress to untreatable HCC if a patient adhered to an annual surveillance program *(33)*. To maximize the utility of an MRI-based screening program for HCC among high-risk individuals, we recommend use of the following overall categories for reporting suspicion of HCC.

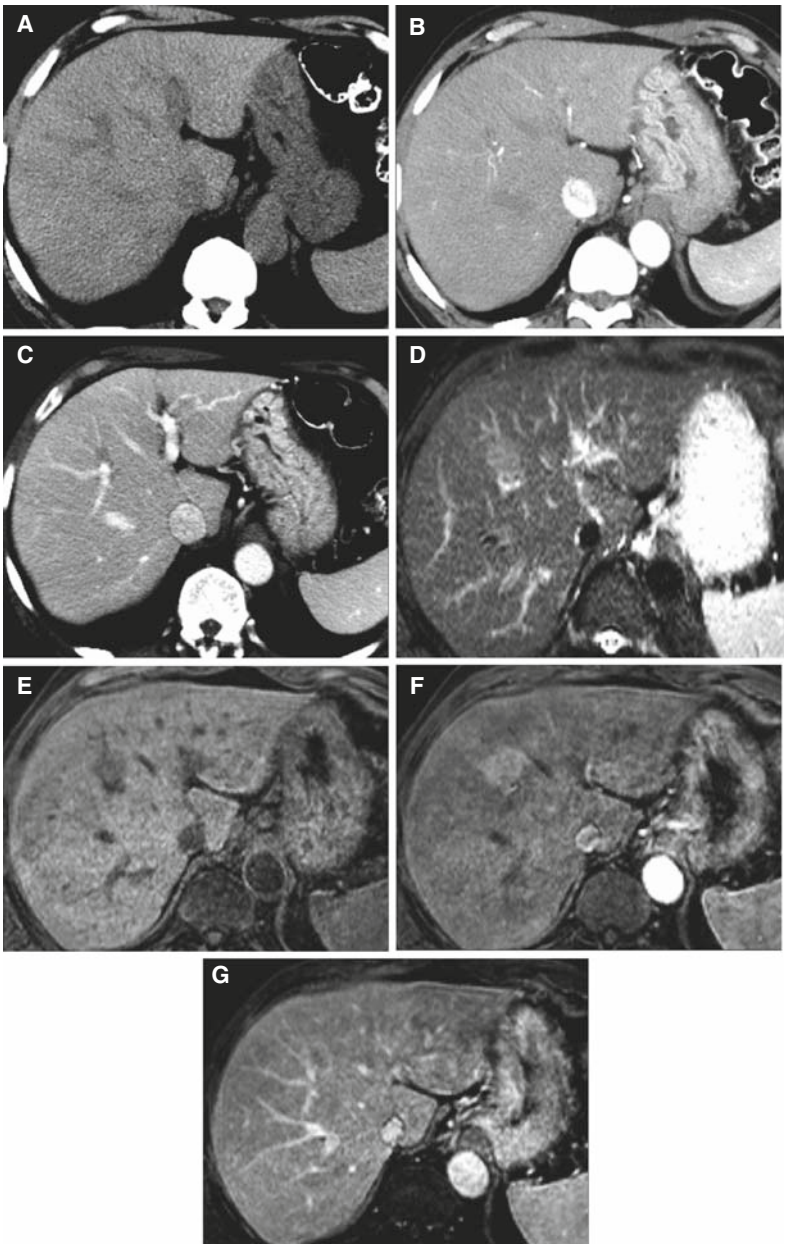

Fig. 3. HCC visible by MRI but not three-phase CT. **A**–**C**. CT images prior to and during arterial and venous phases of contrast enhancement. **D**. T2-weighted MR image shows hyperintense HCC. **E**. T1-weighted MR image shows hypointense HCC. **F**. As in E, immediately after gadolinium contrast agent administration shows moderately hypervascular HCC. **G**. As in E, about 1 minute after gadolinium contrast agent administration does not show the HCC, similar to CT.

4.1. No Nodule with High Suspicion of HCC

These are patients with hepatitis C or other clinical condition that renders them of high risk for developing HCC. This is not changed if low probability lesions, such as subcentimeter foci of transient enhancement, are present. These patients should have repeated examinations at regular intervals, although we are not aware of any data to establish whether 6-month or 12-month intervals are preferable. Although a small minority of subcentimeter transiently enhancing foci may indeed be HCC, well over 90% are benign. If each of these low probability foci triggers a short-term follow-up examination, the overall cost of the screening program may increase geometrically. It is also likely that increasing the frequency of short-term follow-ups may adversely affect overall execution and compliance with the screening program. We therefore recommend that "over-calling" tiny enhancing foci be minimized, provided that these patients are still imaged with a frequency of at least one MRI examination per year *(33)*.

4.2. Indeterminate Nodule

These are usually nodules larger than 1 cm, or other imaging characteristic to generate more than a low probability level of confidence. A diameter of greater than 1 cm is important for two separate reasons. Benign enhancing nodules are usually less than 1 cm in diameter, so larger size by itself raises the possibility of HCC. Additionally, the danger of "under-calling" lesions larger than 1 cm is that tumor doubling will have a more adverse affect if the nodule is already greater than 1 cm. The goal of a screening program is to detect a nodule while it still can be treated optimally. As a mass exceeds 2 cm and becomes progressively larger, the possibility of unsuccessful treatment increases.

An indeterminate nodule will usually trigger a short-term follow-up. The recommended interval will depend on the level of concern regarding rapid interval growth. Typically, the interval recommended will be between 6 and 12 weeks. Alternatively, an ultrasound with potential biopsy may be recommended. If a nodule is visible by sonography as a distinct hypoechoic nodule, this increases the likelihood that it is HCC. Ultrasound may then be used to guide biopsy, if its location renders it accessible. It must be recognized, however, that guided biopsy may be false negative due either to sampling error or to occasional similarity between well-differentiated HCC and benign liver tissue. Therefore, negative results of a biopsy of indeterminate nodule should still trigger short-term imaging follow-up.

4.3. Probable HCC

These will be distinct nodules which are visible on more than one pulse sequence. Their distinction vs. the next category of risk will depend largely on the expertise and experience of the interpreting radiologists, as well as the quality of the MRI examination.

4.4. HCC

For these lesions, the characteristics of HCC are sufficiently clear that there is no reasonable doubt as to the diagnosis. It is becoming standard practice that a confident diagnosis from a reliable radiologist can be used to direct management decisions regarding HCC, in the absence of tissue diagnosis. In some instances, biopsy or documented rising α-fetal protein levels might be insisted upon, such as to list for transplantation, if the nodule is less than 2 cm diameter.

5. CONCLUDING STATEMENTS

Hopefully, the above discussions will help to improve communications between the various physicians involved in managing patients with HCC with regard to their diagnosis by MRI. As official criteria for assigning priority for liver transplant evolve, standards for reporting measurements of size and number may change. Regardless, it is important that all those involved in interpreting MR images and generating their reports are fully cognizant of the affects of these reports on patients' categories for prioritization.

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14 Ultrasound of Hepatocellular Carcinoma: The Important Contribution of Contrast Enhancement

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CONTENTS

ABSTRACT

Ultrasound is established as a screening method in the patient at high risk for hepatocellular carcinoma. US detection of a nodule in such a patient is frequently followed by contrast-enhanced ultrasound (CEUS) performed with the addition of a microbubble contrast agent. This allows for the evaluation of the mass in a similar manner to that on CT and MR scan where liver mass diagnosis is based on the enhancement characteristics of the mass in the arterial and portal venous phases. CEUS plays an integral role with CT and MR scan in the evaluation of the patient at risk for hepatoma. The classic enhancement characteristics of arterial phase hypervascularity and

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portal phase washout are shown in the majority of patients. However, well recognized is arterial phase hypovascularity and lack of washout in the portal phase especially in well-differentiated tumors. The real-time nature of CEUS gives it a unique role in the evaluation of small tumors in particular. Its versatility of performance is also invaluable for monitoring RFA and showing response to therapy.

Key Words: Ultrasound; CEUS (contrast-enhanced ultrasound); hepatocellular carcinoma; dysplastic nodule; screening

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy. The majority of HCCs occur on a background of liver cirrhosis and it is now well recognized that HCCs commonly develop through a multistep carcinogenesis from low-grade dysplastic nodule (DN), high-grade DN, well-differentiated HCC to classic advanced HCC *(1, 2)*. The change of histologic types of the nodule is believed to be sequential; however, the distinctions between each step are not always clear even on histopathology, which suggests a continuous transition *(1)*. HCCs may also arise de novo from a relatively normal liver without a background of RN or DN, especially in non-Asian populations *(3)*. The early diagnosis of HCC is important since the treatment is most effective when the tumor is small.

Detection and characterization of HCC is one of the major roles of imaging diagnosis in high-risk patients. Imaging diagnosis of HCC is primarily based on sequential changes in the intranodular blood supply during the hepatocarcinogenesis; RN show similar blood supply to normal liver, borderline lesions show wide variations of blood supply, and typical HCCs are supplied by abnormal arteries alone *(4)*. Over the last few decades there has been remarkable progress of imaging techniques for diagnosing HCC. The improvement of gray-scale ultrasound (US) scan enables us to detect subcentimeter lesions in the liver. Recent fast computed tomography (CT) and magnetic resonance (MR) scanners provide multi-phasic contrast-enhanced imaging, which has become an integral part of imaging of HCC. Arterial phase (AP) imaging is extremely important to detect and characterize focal liver lesions in a cirrhotic liver. Worldwide, US scan is most commonly used as a screening test for HCC in high-risk patients *(5)*. Multi-phasic contrast-enhanced CT and MR scans are usually performed when there is any focal lesion suspected to be HCC on US or there is a strong clinical suspicion of HCC. However, the pattern of the use of imaging tests is variable depending on each institutional preference. Clinical use of microbubble contrast agents

has expanded the role of US from that of detection to one-stop characterization of HCC based on the enhancement features at contrast-enhanced US (CEUS) *(6–13)*. Current low-mechanical index (MI) techniques for CEUS using second-generation microbubble agents have further advantages in characterizing HCC, including real-time demonstration of continuous hemodynamic changes in both the liver and the liver lesion. In our institution, US scan is used as a routine screening/surveillance imaging test for high-risk patients for HCC, and CT/MR scan and/or CEUS is performed to characterize any focal lesions detected on US scan. A large number of CEUS examinations are also performed to characterize small indeterminate focal liver lesions seen on CT or MR scans, producing satisfactory results. We have recently developed a systematic imaging work-up algorithm to evaluate newly detected liver nodules in screening/surveillance imaging examinations based on the American Association for the Study of Liver Diseases (AASLD) guidelines *(5)*. The algorithm includes performance of CEUS, CT, and MR in all newly detected 10–20 mm nodules and is well received by referring hepatologists.

Although recent progress of imaging techniques improves the sensitivity to detect HCC, it also reveals a large number of pseudolesions and benign tumors that can mimic the appearance of HCC *(15)*. These lesions can alter the management of the patient, potentially preventing curative surgery. It is, therefore, critical to achieve noninvasive characterization of focal liver lesions with reasonable imaging criteria and adequate additional or follow-up imaging studies. Unfortunately, there are significant overlaps between the imaging findings of benign and malignant liver lesions in cirrhotic livers.

In this chapter, we review the typical US and CEUS imaging features of HCC and other cirrhosis-related nodules. We focus, in particular, on the issue of characterization of small liver lesions in the cirrhotic liver. We also discuss the role of US and CEUS in routine screening/surveillance of HCC in high-risk patients and in monitoring therapeutic responses to local ablation therapy or anti-angiogenic agents.

2. GRAY-SCALE AND DOPPLER ULTRASOUND

Liver cirrhosis is a diffuse process of alteration of the normal liver architecture into fibrosis with development of regenerating nodules (RN) throughout the liver. RN do not usually stand out on imaging, but may be seen as ill-defined tiny nodules within a diffusely coarse liver parenchyma on US scan.

Dysplastic nodules (DN) demonstrate variable echogenicity patterns, including hyperechoic, isoechoic, and hypoechoic. Hyperechogenicity is related to the fatty metamorphosis that may be seen in DN *(15)*. The internal

architecture of DN is usually homogeneous; however, it is impossible to differentiate DN from small HCC by gray-scale US findings alone.

HCC may grow as solitary or multiple discrete nodules or show as an ill-defined infiltrative mass. It is usually easy to make a diagnosis of HCC when the tumor is large if expansive or advanced infiltrative tumors are shown. Expansive HCCs are well demarcated, nodular, and frequently encapsulated (Fig. 1). On the other hand, infiltrative HCCs have irregular and indistinct margins (Fig. 2) with frequent invasion of the portal veins or hepatic veins *(2)*. A mixed expansive and infiltrative growth pattern is not uncommon. Expansive HCCs usually have a better prognosis and better response to treatment.

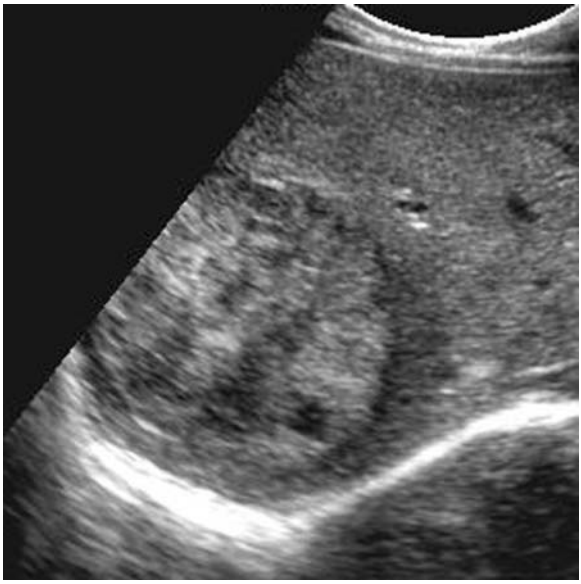

Fig. 1. Encapsulated HCC in a 53-year-old man with hepatitis B. US image shows a large heterogeneous mass surrounded by a hypoechoic rim, representing a fibrous pseudocapsule.

Fig. 2. Infiltrative HCC in a 49-year-old man with hepatitis B. (**a**) US image shows an ill-defined hypoechoic mass (*arrows*) in the liver. (**b**) CEUS scan in the arterial phase at 10 s after injection of the contrast material shows diffuse hypervascularity within the mass with an irregular margin. (**c**) CEUS image at 134 s after injection of the contrast material shows negative enhancement (washout) of the mass relative to the liver. (**d**) CT image in the arterial phase shows a hyperenhancing mass with an irregular margin. (**e**) The mass shows washout in the portal venous phase.

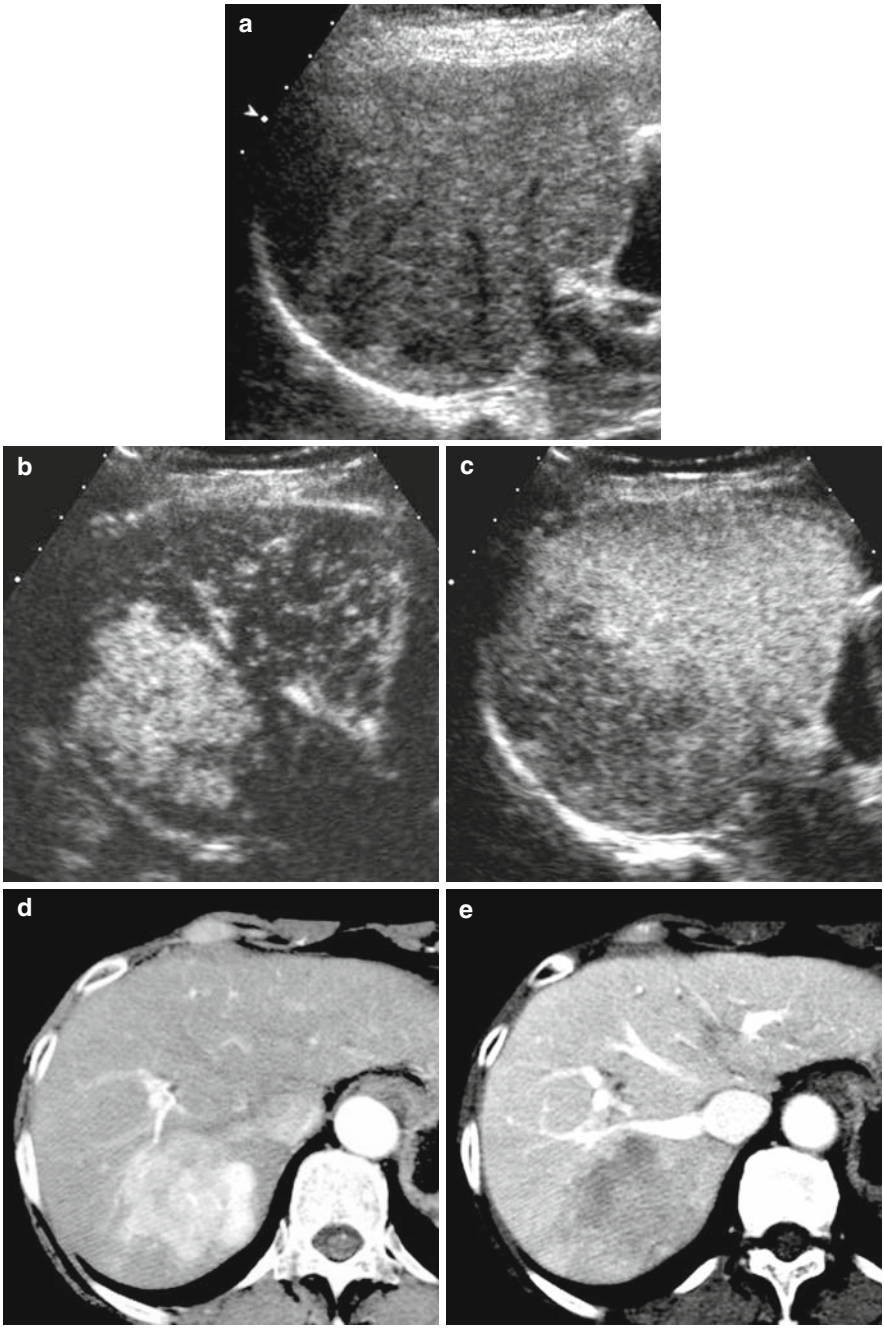

Fig. 2. (Continued)

HCCs have variable echogenicity on gray-scale ultrasound. Small tumors without fatty metamorphosis are usually hypoechoic, but the echo pattern changes as the size increases. Small HCCs with fatty metamorphosis typically show hyperechogenicity, potentially mimicking the appearance of hemangioma on gray-scale US *(16)*. With time and increasing size, the masses tend to become more complex and inhomogeneous as a result of necrosis. HCC with expansive growth is usually seen as a discrete nodule

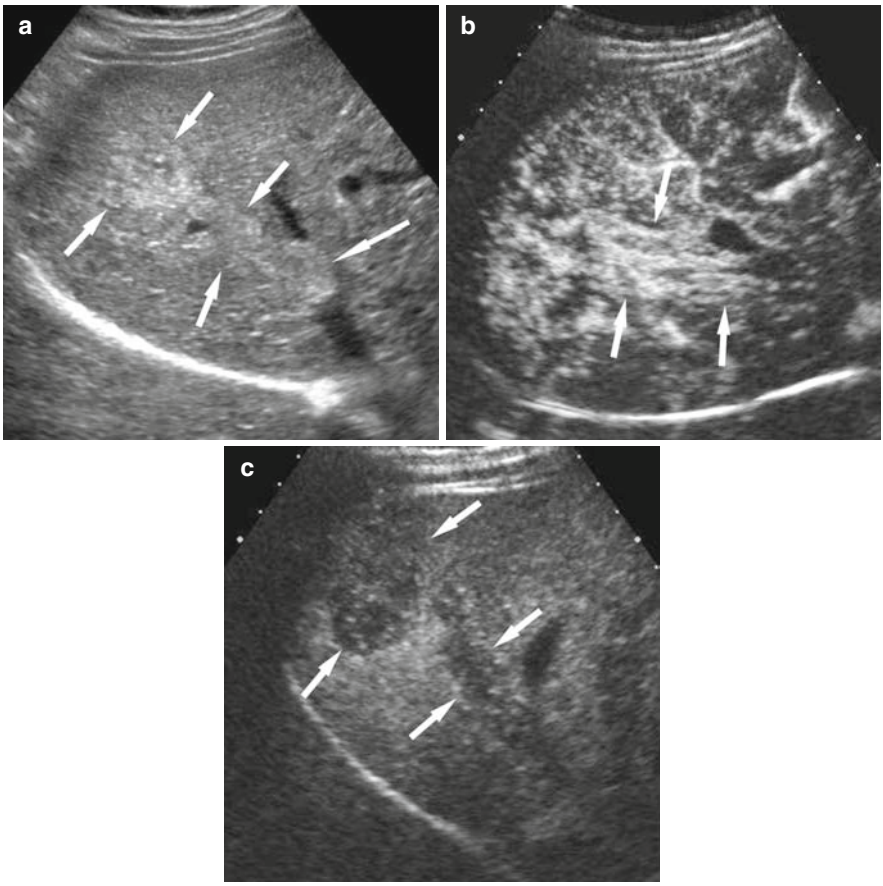

Fig. 3. Infiltrative HCC with right hepatic vein thrombosis in a 42-year-old man with hepatitis B. (**a**) US image shows an ill-defined slightly hyperechoic area (*arrows*) in the liver. There is a focal thrombosis (*long arrow*) in the right hepatic vein. (**b**) CEUS image in the arterial phase at 9 s shows heterogeneous hypervascularity of the lesion with linear enhancing structures along the course of right hepatic vein thrombosis (*arrows*). (**c**) CEUS image at 247 s shows washout of the mass and right hepatic vein thrombus (*arrows*).

with heterogeneous echo texture and frequently has a hypoechoic peripheral halo which corresponds to a fibrous pseudocapsule (Fig. 1) *(17)*. An uncommon but characteristic appearance of HCC is a nodule-in-nodule pattern which represents a focus of HCC within a DN or areas of different degrees of differentiation of HCC. In contrast, infiltrative HCC appears as an area with heterogeneous echogenicity and can be easily overlooked on an US scan. It is important to carefully evaluate portal or hepatic vein branches within any suspicious heterogeneous area because tumor thrombosis is frequently associated with infiltrative HCC (Fig. 3). Intratumoral fat also occurs in larger HCC. Because it tends to be focal, however, it is unlikely to cause confusion in diagnosis. Rare surface lesions may present with spontaneous rupture and hemoperitoneum.

Fibrolamellar carcinoma is a histologic subtype of HCC that is found in younger patients (adolescents and young adults) without coexisting liver disease. The serum α-fetoprotein levels are usually normal. The tumors are usually well differentiated, often encapsulated by fibrous tissue and solitary. The prognosis is generally better for fibrolamellar carcinoma compared with typical HCC. Most patients, however, demonstrate advanced disease at the time of diagnosis *(18)*. The echogenicity of fibrolamellar carcinoma is variable. Punctuate calcification and a central echogenic scar—features which are distinctly unusual in HCC—are more common in the fibrolamellar subtype.

Color or power Doppler US scan typically shows high-velocity arterial flow within large HCC (Fig. 4). A pattern analysis of the distribution of intratumoral flow might be helpful to suggest the diagnosis of HCC *(19–21)*;

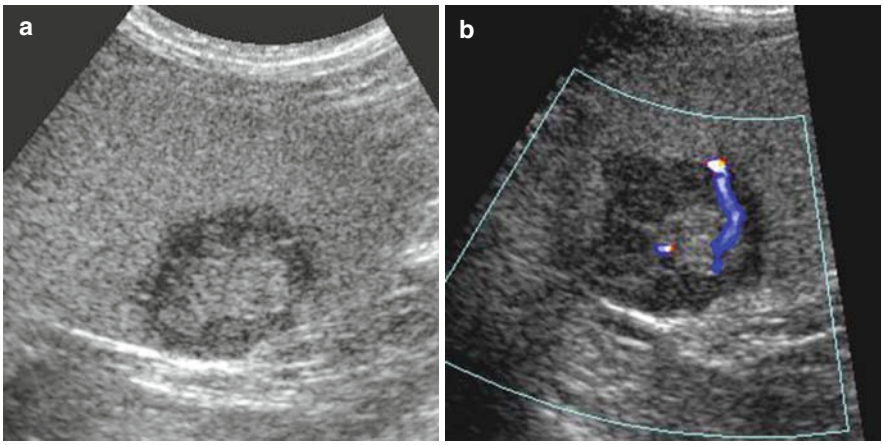

Fig. 4. HCC in a 81-year-old man with hepatitis C. (**a**) US image shows a well-defined hypoechoic mass in the liver. (**b**) Color Doppler US image shows a linear intratumoral vessel showing arterial flow on spectral Doppler examination (not shown).

however, it is rarely specific and requires a further imaging test for confirmation. Doppler is excellent for detecting neovascularity within tumor thrombi in the portal veins, diagnostic of hepatocellular carcinoma even in the absence of demonstration of the parenchymal lesion (Fig. 5).

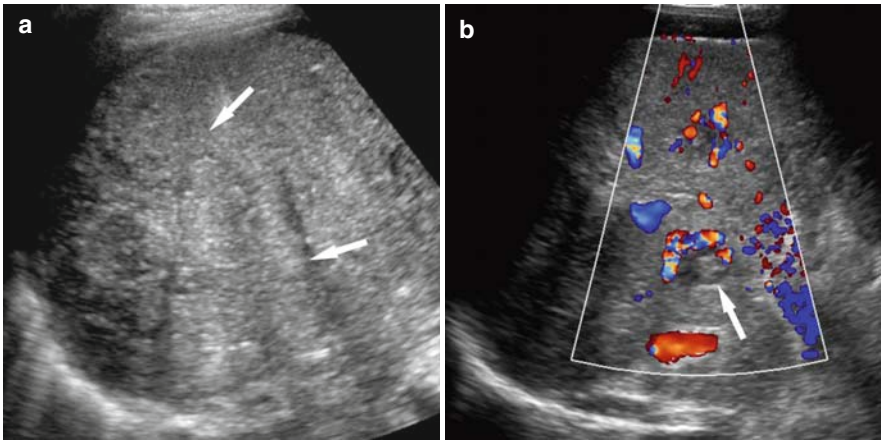

Fig. 5. Infiltrative HCC with right portal vein thrombosis in a 51-year-old man with hepatitis C. (**a**) US image shows an ill-defined heterogeneous area (*arrows*) in the liver. (**b**) Color Doppler US image shows a thrombosis within the right portal vein containing pulsating flow representing tumor thrombosis.

3. CONTRAST-ENHANCED ULTRASOUND

3.1. Techniques

US contrast agents, which are presently used in radiology, consist of microbubbles of perfluorocarbon gas stabilized by a protein, lipid, or polymer shell. The bubbles are sufficiently small and stable to traverse the pulmonary and cardiac circulations following peripheral venous injection. The bubbles disappear as the gas diffuses through the thin shell, with a typical half-life of a few minutes in blood. In our experience with more than 4,000 injections, patient acceptance has been very high, with no serious adverse events seen at our institutions. A large retrospective study from Europe using a slightly different type of microbubble contrast agent reported 0.0086% incidence of serious adverse events without any fatality among 23,188 examinations *(22)*. The bubbles are approximately the same size as red blood cells and cannot move through the vascular endothelium into the interstitium, even after an extended period of time; therefore, they are true blood pool agents *(23)*. Microbubble contrast agents are approved for radiologic use in many countries, including the European Union, Canada, and many Asian countries. Although US contrast material has been approved for clinical use

for cardiac diagnosis in the United States for a number of years, its use for radiologic indications is still under investigation at the time of writing this chapter.

A contrast-specific imaging mode, such as pulse inversion technology, is available on all high-end US systems and is essential for the visualization of microbubbles. The use of low-mechanical index (MI) imaging is critical for continuous, real-time evaluation of enhancement. Typically, the contrast agent is injected manually through a three-way stopcock, followed by a 5-mL saline solution flush. Low-MI continuous imaging is performed during the arterial and portal venous phases. Slightly higher MI and larger amounts of microbubbles can be used for deep-seated lesions or lesions within an attenuating liver. The first injection usually includes a stationary field of view to include the lesion of interest and adjacent liver, both observed continuously for 4–5 min. Subsequent injections concentrate on arterial phase vessel morphology and enhancement as well as sweeps of the entire liver in the portal venous phase to look for any further abnormalities. Injections are typically repeated two to five times to obtain images for the same lesion or to evaluate a different lesion. Each injection is separated by 3–5 min. A simultaneous dual-imaging mode, which displays gray-scale imaging and contrast-specific imaging side-by-side, is available on most updated ultrasound scanners. The dual-imaging mode is particularly useful to evaluate small liver nodules. A flash-replenishment technique in conjunction with real-time maximum intensity processing is useful to characterize vascular patterns and morphology of the vessels in the arterial phase *(24)*.

3.2. Differential Diagnosis of Nodules in Liver Cirrhosis

Presently, the evaluation of the blood supply in a hepatocellular nodule is the single most important imaging parameter to characterize nodules in liver cirrhosis, because there are sequential changes in the supplying vessels and hemodynamic state during hepatocarcinogenesis *(25)*. Clinical use of microbubble contrast agents enables US to characterize HCC based on the enhancement features. Current real-time low-MI imaging techniques with second-generation contrast agents have remarkably improved the capability of CEUS in the characterization of HCC and their differentiation from various nodules related to cirrhosis. It is now feasible to focus on a small indeterminate nodule from wash-in to washout of contrast and CEUS can provide better understanding of complex hemodynamic changes of a nodule and a cirrhotic liver.

Classic HCCs are typically supplied by abnormal arteries alone and show positive enhancement during the hepatic arterial phase and negative enhancement (washout) during the portal venous phase *(11–13)*. There are irregular dysmorphic arteries within the tumor often visualized in large HCC in the early arterial filling phase (Fig. 6). Detection of arterial

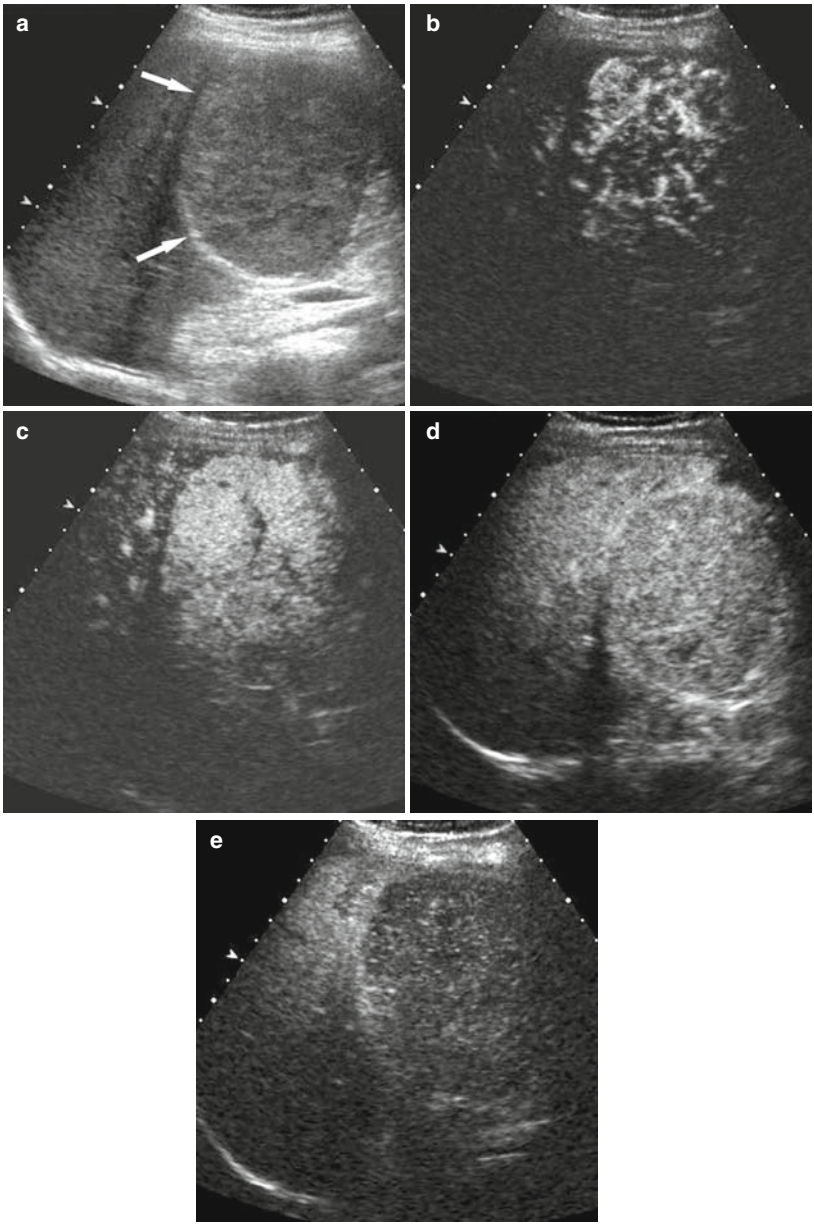

Fig. 6. Typical HCC in a 38-year-old man with autoimmune hepatitis. (**a**) US image shows a large well-defined hypoechoic mass (*arrows*) in the liver. (**b**) CEUS scan in the arterial phase at 6 s shows dysmorphic arteries within the mass. (**c**) CEUS scan at 12 s shows homogeneous enhancement of the mass with small non-enhancing necrotic areas. (**d**) CEUS image at 69 s shows slight washout of the mass relative to the liver. (**e**) The mass shows clear washout at 144 s after injection.

hypervascularity is very important to make a diagnosis of HCC as it is one of the most reliable characteristics of nodular HCC. However, there is a small subset of HCC with no arterial hypervascularity, including particularly those that are well differentiated *(26)*. CEUS is also excellent in the differentiation between tumor thrombosis and benign thrombosis in the portal vein. Tumor thrombi invariably show heterogeneous enhancement and linear, irregular feeding vessels after injection of the microbubbles (Fig. 7) whereas benign thrombi are avascular.

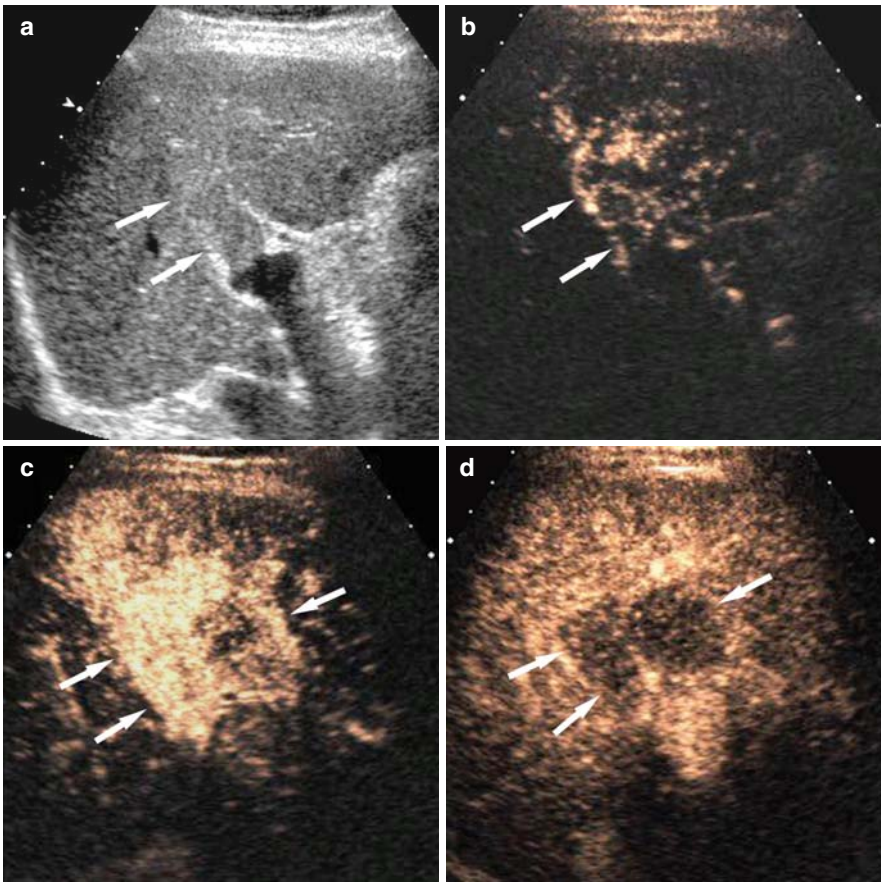

Fig. 7. HCC with portal vein thrombosis in a 58-year-old man with hepatitis C. (**a**) US image shows an expanding thrombosis in the right portal vein (*arrows*). (**b**) CEUS image in the arterial phase at 6 s shows linear arteries along the thrombosed portal vein branching into the tumor thrombi (*arrows*). (**c**) CEUS image at 14 s shows homogeneous enhancement of the mass and tumor thrombi (*arrows*) in the right portal vein. (**d**) CEUS image at 86 s shows washout of the mass and right portal vein thrombi (*arrows*) relative to the liver.

Negative enhancement or ‘washout’ during the venous phase is also an important characteristic of HCC as typical tumors lack portal venous supply. The intensity of enhancement of HCC in the portal venous phase, however, generally decreases more slowly than that in a metastasis. In our study of 115 hypervascular HCC *(26)*, only 50% showed the expected portal phase washout by 90s. Extended evaluation over 3 min is important to characterize HCC by demonstrating ‘eventual’ washout (Fig. 8). Further, sustained positive enhancement in the extended portal phase should not be considered diagnostic of a benign lesion, especially in patients at risk for HCC since it may occur in well-differentiated HCC (Fig. 9).

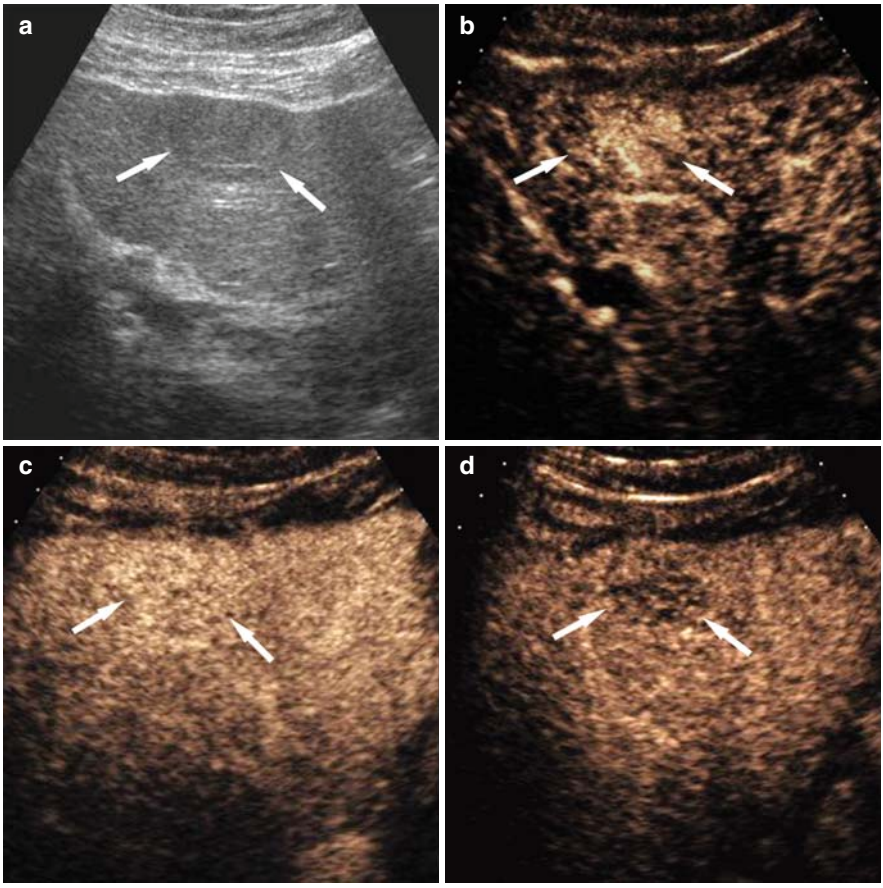

Fig. 8. HCC with late washout in an 85-year-old man with hepatitis C. (**a**) US image shows a hypoechoic mass (*arrows*) in the liver. (**b**) CEUS scan in the arterial phase at 8 s shows heterogeneous hypervascularity of the mass (*arrows*). (**c**) CEUS image at 133 s shows isoechogenicity of the mass (*arrows*) relative to the liver. (d) CEUS image at 213 s shows washout of the mass (*arrows*).

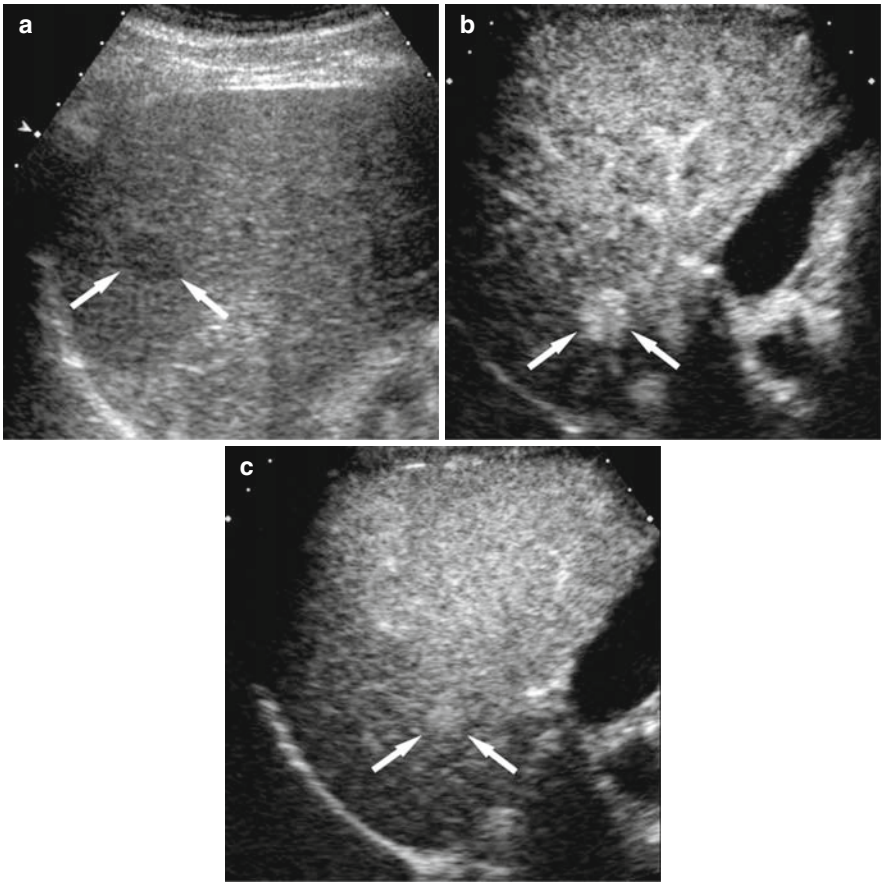

Fig. 9. HCC with no washout in a 61-year-old man with hepatitis B. (**a**) US image shows a hypoechoic nodule (*arrows*) in the liver. (**b**) CEUS scan in the arterial phase at 21 s shows homogeneous hypervascularity of the nodule (*arrows*). (**c**) CEUS image at 218 s shows persistent hyperechogenicity of the nodule (*arrows*) relative to the liver.

Most RN are isoechoic to the parenchyma during all phases on CEUS (Fig. 10), although they may show transient hypovascularity in the arterial phase. As DN have more histological atypia, abnormal arteries increase while normal arterial and portal supply decrease. The arterial and portal supplies to DN are variable and inconsistent (Fig. 11) *(27)*. Moreover, there are significant overlaps of vascular supply between DN and well-differentiated HCC. CEUS, CT, and MR all suffer from similar problems in the imaging of these nodules. CEUS may have advantages from continuous observation in detecting subtle vascular differences of HCC from DN.

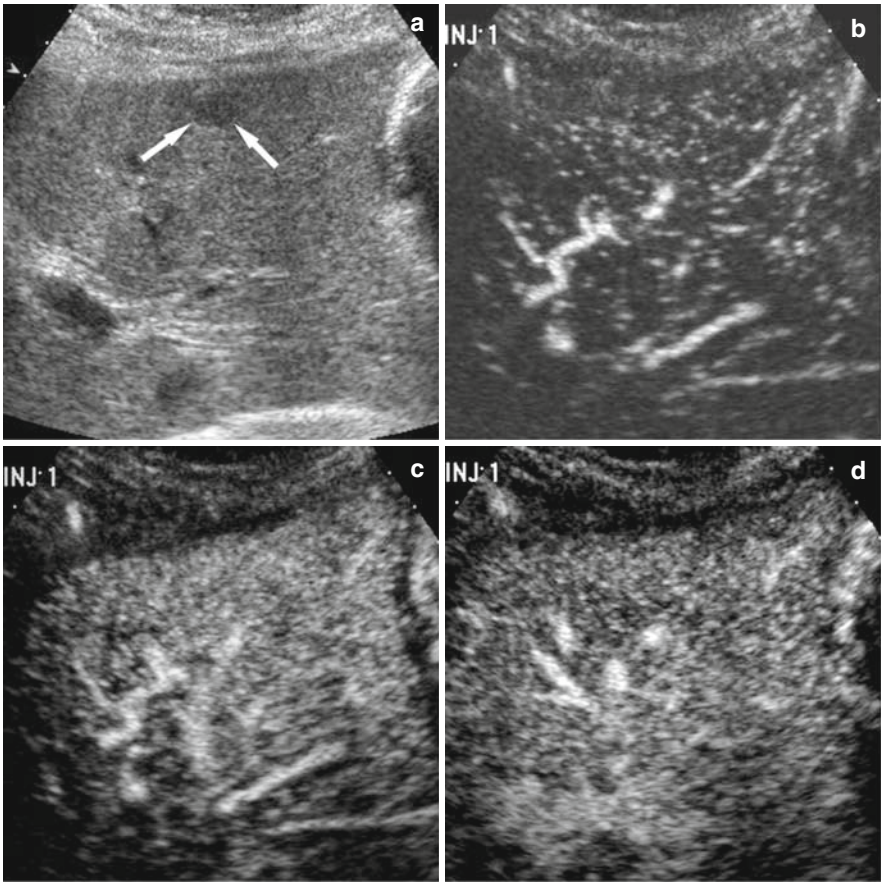

Fig. 10. Regenerative nodule in a 52-year-old man with hepatitis B. (**a**) US image shows a hypoechoic nodule (*arrows*) in the liver. (**b–d**) The nodule is not visualized on CEUS scans at 22 s (**b**), 31 s (**c**), and 113 s (**d**) after injection of the contrast material because of isoechogenicity of the relative to the liver.

3.3. Role of US and CEUS in HCC Surveillance

Surveillance for HCC in high-risk patients is widely practiced particularly in endemic regions such as East Asia. A recent practice guideline for the management of HCC by the American Association for the Study of Liver Diseases (AASLD) recommended that surveillance for HCC should be performed using US at 6–12 month intervals *(5)*. Traditionally, the diagnostic confirmation of HCC was made by tumor biopsy. However, there is a recent trend to diagnose typical cases of HCC based on imaging and clinical findings without biopsy. For example, the AASLD guideline recommended that the diagnosis of HCC can be made without biopsy in patients

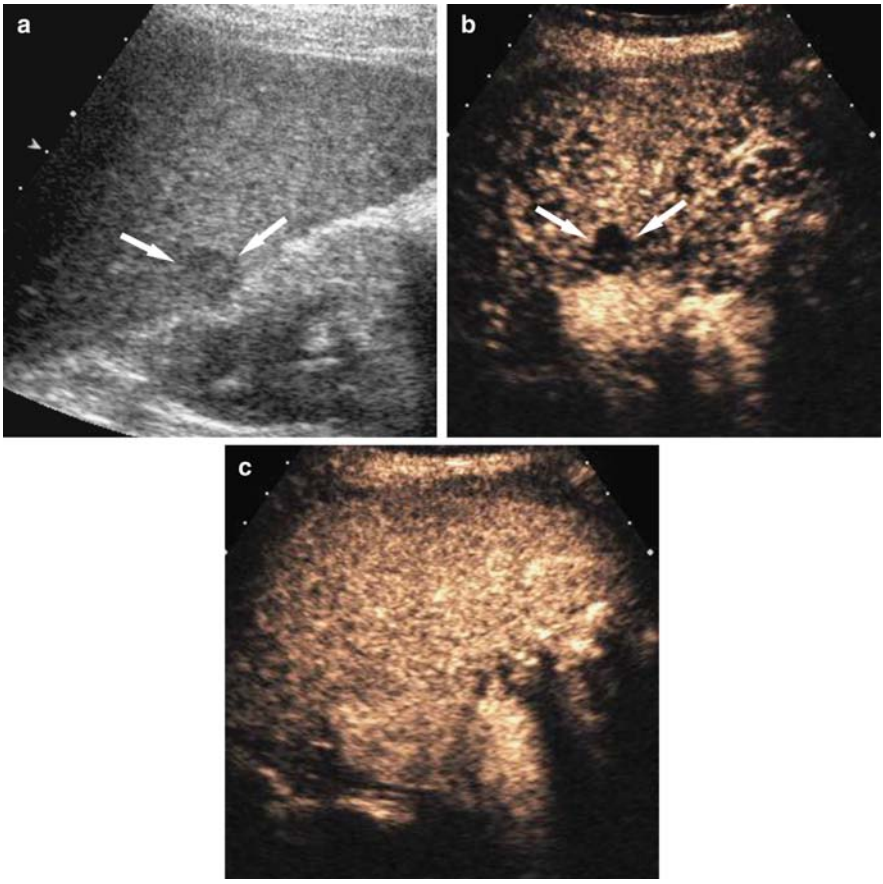

Fig. 11. Dysplastic nodule in a 49-year-old woman with hepatitis B. (**a**) US image shows a hypoechoic nodule (*arrows*) in the liver. (**b**) CEUS image in the arterial phase at 10 s shows hypovascularity of the nodule (*arrows*) relative to the liver. (**c**) The nodule is not visualized on CEUS scan at 168 s because of isoechogenicity.

with cirrhosis with typical enhancement patterns of HCC on one dynamic contrast-enhanced imaging technique for lesions larger than 2 cm and on two dynamic imaging studies, including multi-phasic contrast-enhanced CT, MR, or CEUS, for lesions between 1 and 2 cm *(5)*. This guideline defines typical enhancement pattern of HCC as hypervascularity of the lesion in the arterial phase and negative enhancement (washout) of the lesion relative to the hepatic parenchyma in the portal venous or delayed phase.

The University Health Network in Toronto recently developed a systematic imaging work-up protocol for newly detected nodules on surveillance US. Our experience for the initial 2 years shows that surveillance

US is able to detect small lesions (<2 cm) in the majority of cases, and a multi-modality imaging approach with contrast-enhanced CT, MR, and CEUS provides an excellent diagnostic ability to characterize typical HCC even if they are smaller than 2 cm. However, there are still considerable numbers of indeterminate lesions in 1–2 cm nodules requiring biopsy. Our experience also shows that about one-fourth of newly detected lesions are hemangiomas and those cases are easily characterized by CEUS at the time of detection, preventing extensive imaging work-up, additional hospital visits, and invasive procedures (Fig. 12).

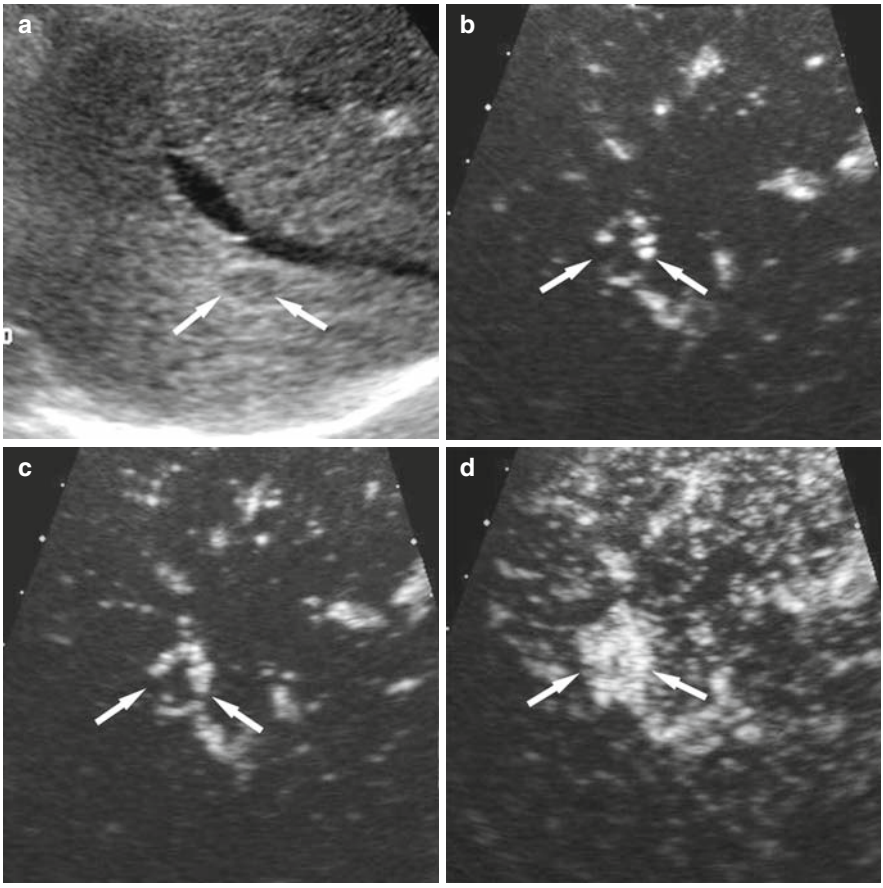

Fig. 12. Hemangioma in a 52-year-old woman with hepatitis C. (**a**) US image shows a slightly hypoechoic nodule (*arrows*) in the liver. (**b**–**d**) CEUS images at 6 s (**b**), 7 s (**c**), and 10 s (**d**) after injection of the contrast material show peripheral nodular enhancement of the nodule (*arrows*) with subsequent central fill-in. The nodule shows homogeneous hyperechogenicity at 10 s (**d**).

Real-time CEUS is excellent in the characterization of hemangiomas, regardless of the rapidity of the enhancement *(28)*.

3.4. Post-treatment Monitoring of HCC

Radiofrequency ablation (RFA) has become one of the main treatment modalities for patients with small HCC. Real-time gray-scale US scan is most frequently used for the guidance for RFA procedure; however, there are uncommon cases with poor visibility on US scan. CEUS can be extremely helpful to localize the lesion by demonstrating the arterial phase hypervascularity and washout in the portal venous phase. The use of dual-imaging mode, which displays gray-scale imaging and contrast-specific imaging side-by-side, is critical to visualize the lesion and the needle simultaneously. A routine use of pre-procedure CEUS can reduce the number of incomplete or erroneous RFA significantly.

On the other hand, an accurate assessment of the RFA therapeutic response is crucial because a complete tumor ablation significantly improves patient survival, whereas residual or recurrent HCC may immediately require an additional treatment. CEUS can be applied immediately after RFA procedure so that repeated RFA can be carried out immediately after the procedure in the same treatment session if residual enhancing tumor is found *(29*, *30)*. Contrast-enhanced CT or MR imaging is most commonly used for interval post-RFA monitoring, but CEUS can be used as a useful alternative or a problem-solving method when CT or MR imaging is not conclusive. On CEUS, successful treatment means complete avascularity within the treated HCC. Any intratumoral enhancement indicates residual viable HCC and requires additional RFA procedure (Fig. 13). Benign perfusion abnormalities adjacent to the ablation zone are frequently seen and may persist several months after RFA procedure. It is, therefore, important to define the outer border of the pre-existing tumor and assess any enhancing area within the border. Ill-defined hypervascular areas outside the border usually represent benign perfusion abnormalities and these areas do not show washout in the portal venous phase.

4. CONCLUSION

Recent advances in liver imaging techniques and better understanding of imaging findings have facilitated the detection and characterization of hepatocellular nodules in a cirrhotic liver. It is important to recognize that various types of benign nodules and pseudolesions are identified on all imaging scans performed for the diagnosis of HCC. An accurate differentiation between them is critical for adequate management of patients with cirrhosis. Unfortunately, any of the imaging tests and even percutaneous biopsy

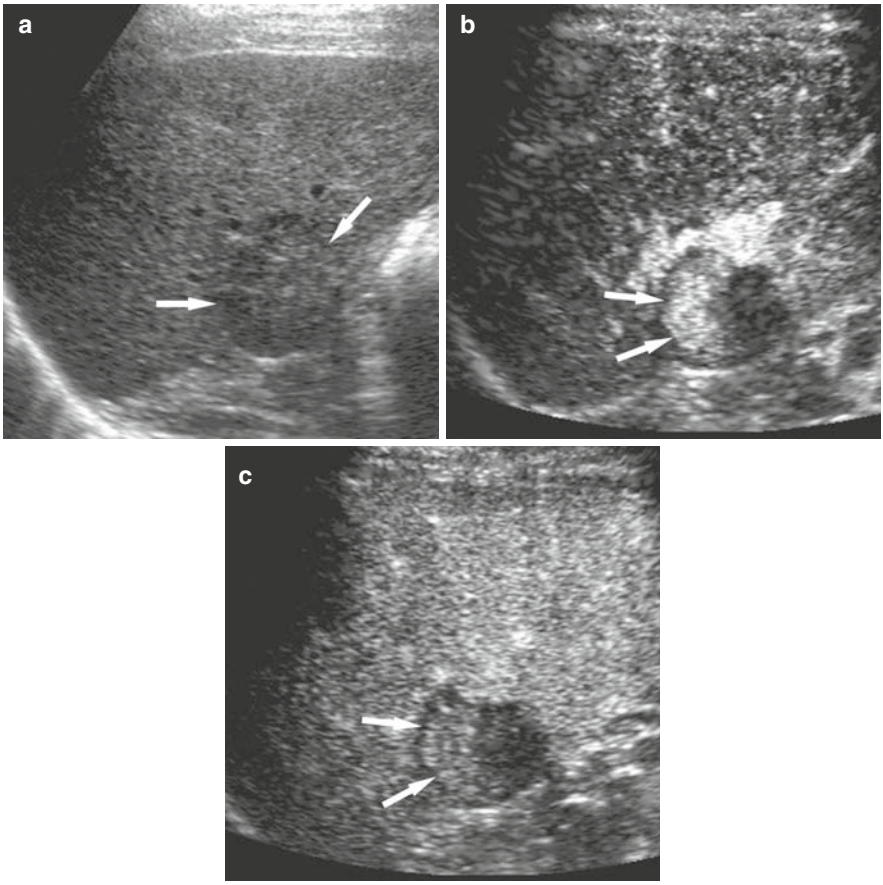

Fig. 13. Residual viable tumor after radiofrequency ablation for HCC in a 54-year-old woman with hepatitis B. (**a**) US image shows a hypoechoic mass (*arrows*) which has been treated with radiofrequency ablation. (**b**) CEUS image in the arterial phase at 11 s shows an eccentric intratumoral area of hypervascularity (*arrows*), representing residual viable tumor. (**c**) The intratumoral enhancing area (*arrows*) shows washout at 59 s.

is not diagnostic for borderline lesions. Intimate collaboration of hepatologists, pathologists, surgeons, and radiologists with reasonable imaging and clinical criteria estimating the degree of malignancy is imperative.

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15 Percutaneous Ethanol Injection

Tito Livraghi MD, Maria Franca Meloni MD, and Anita Andreano MD

CONTENTS

ABSTRACT

The chapter considers the principles, the techniques, the results of PEI for treating cirrhotic patients with HCC, and its current indications compared to those of RF, which is now considered the gold standard.

HCC is an organ pathology, so the first nodule detected is only a prelude to others. Therefore, hepatic resection or percutaneous ablation therapies can offer a palliative cure, achieving only a local control of the disease. Although it is understood that surgery assures the highest possibility to completely ablate the tumor and the possible satellites, recent RCTs comparing resection and percutaneous ablation therapies demonstrated roughly equivalent results.

As radiofrequency is actually considered the gold standard ablation technique, the current place of PEI has to be determined. Of course where radiofrequency is not available PEI remains a valid treatment for HCC, especially for health-care systems with limited economical resources. Moreover

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in all those cases in which radiofrequency is considered to be at risk for complications, PEI is a valid alternative, i.e., in case of lesions adjacent to main biliary ducts or to intestinal loops. PEI is also useful to treat lesions close to large vessels, as it is not affected by the so-called sink effect. PEI remains a good indication to treat segmental portal thrombosis.

Key Words: Percutaneous Ethanol Injection (PEI); Percutaneous Ablation Therapy (PAT); Radiofrequency (RF); Transarterial chemoembolization (TACE); Contrast-Enhanced Ultrasound (CEUS); Hepatocellular Carcinoma (HCC); Hepatic Resection (HR)

1. INTRODUCTION

Percutaneous ablation therapies (PATs) of hepatic neoplasms are performed using an image-guided approach through the liver parenchyma. PATs may be based on the use of means capable of destroying the tissue chemically, such as ethyl alcohol (PEI) or acetic acid (PAI), or physically, as with laser (ILP), radiofrequency (RF), or microwave (MW). PEI, the first of PATs to be proposed, was independently conceived at the University of Chiba in Japan and at the Vimercate Hospital (Milan) in Italy. The first study in an international journal appeared in 1986 *(1)*. On the basis of its rationale and the results obtained, the other techniques were subsequently designed *(2–5)*. The range of indications for PATs is currently wider compared to its initial use. Indeed, whereas for some years only patients with up to three small (max. 3 cm in size) or single (max. 5 cm in size) lesions were treated, with the introduction of the "single-session" procedure under general anesthesia *(6)*, even patients with lesions greater in number or larger in size could have been treated. This chapter considers the principles, the techniques, the results of PEI, and its current indications compared to those of RF, which is now considered the gold standard.

2. PRINCIPLES AND TECHNIQUES

PEI is generally performed under ultrasound (US) guidance, because real-time control allows faster execution, precise centering of the needle into the target, continuous monitoring of ethanol distribution, and determination of the appropriate amount of ethanol to be injected each time. The material to perform the procedure is very poor, consisting of a siring, a multihole 22 G needle, and a phial of 95% ethanol (Fig. 1). Alcohol acts by two mechanisms. The first is due to its diffusion within the cells, which causes immediate dehydration of cytoplasmic proteins with

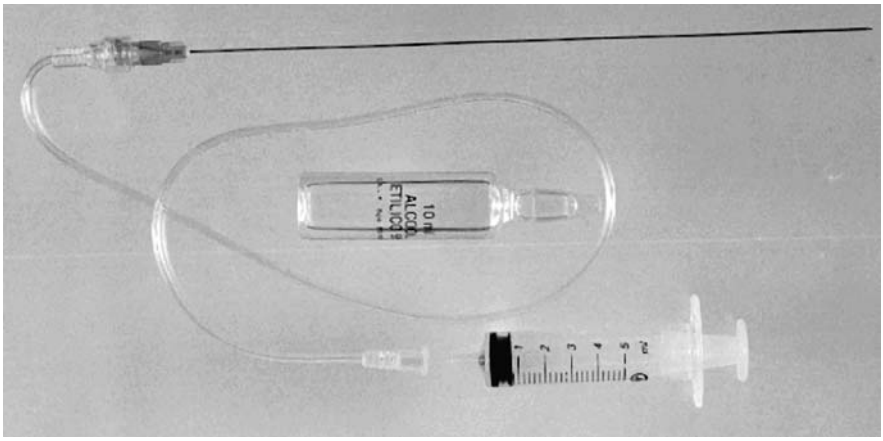

Fig. 1. Material used to perform PEI.

consequent coagulation necrosis followed by fibrosis. The second is due to its entrance in the circulation, which induces necrosis of endothelial cells and platelets aggregation with consequent thrombosis of small vessels followed by ischemia of the neoplastic tissue. Two characteristics of HCC favor the toxic action of ethanol: hypervascularization and difference in consistence between neoplastic and cirrhotic tissue. Since the neoplastic tissue of HCC is softer than the surrounding cirrhotic tissue, ethanol diffuses within it easily and selectively, whereas at the same time hypervascularization facilitates its uniform distribution within the rich network of neoplastic vessels. On the contrary, ethanol diffusion can be impaired in the presence of septa or even impossible in the presence of satellites because of the interposition of cirrhotic tissue *(7)*.

Conventional PEI is performed in multiple sessions on outpatient setting or, when the tumor is more advanced, in a "single session" under general anesthesia with the patient being hospitalized. The former technique is generally used for single HCC <4–5 cm in diameter or for multiple HCC with 2–3 nodules ≤3 cm in diameter. The number of sessions is approximately twice the diameter of the lesion in centimeters *(8)*. The latter technique is adopted for more advanced HCC, single or multiple, that does not involve more than 30% of the hepatic volume and with no neoplastic thrombosis in the main portal branches or in the hepatic veins *(9)*. PEI can also be performed in selected patients with segmental or subsegmental portal thrombosis, injecting 1–3 ml of ethanol directly into the thrombus *(10)*. More detailed technical information about the procedures are available in several studies *(7–12)*.

Recently the use of a multipronged needle to treat medium to large HCC has been proposed. However, there is concern about its safety as

inserting this kind of needle is more technically demanding compared to the conventional one and placing any of its tines outside the tumor can cause alcohol spill, increasing the risk of complications *(13)*.

3. EVALUATION OF THERAPEUTIC EFFICACY

To evaluate the therapeutic response, that is, to determine whether the tumor has become completely necrotic or whether areas of neoplastic tissue are still present, a combination of investigations and serum assays for tumor

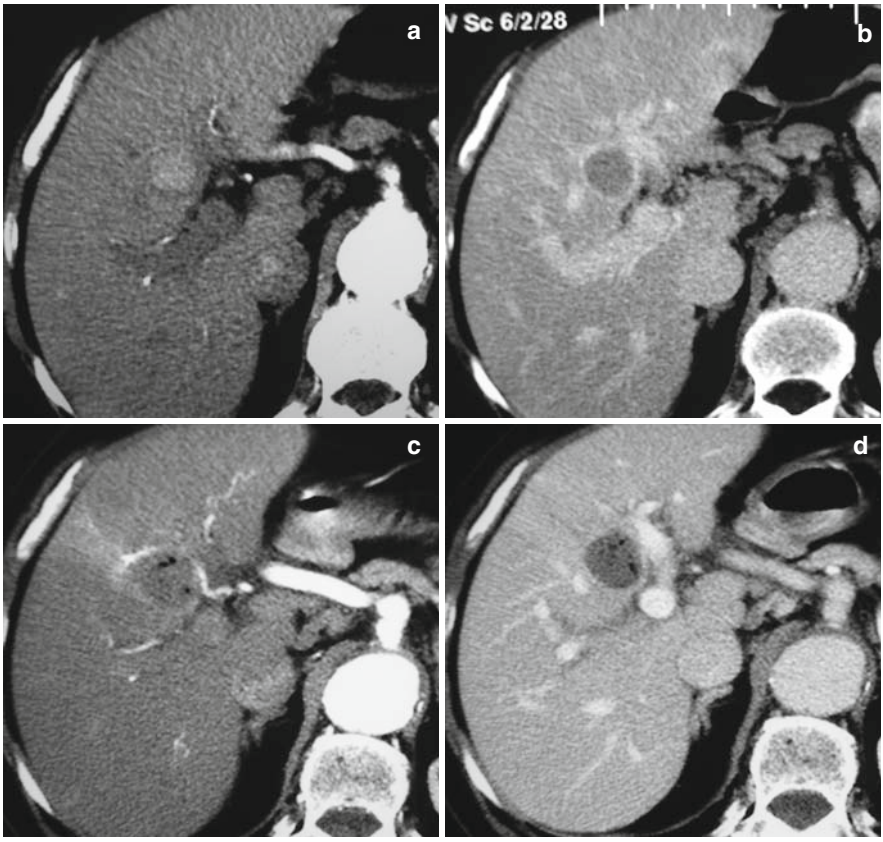

Fig. 2. Transverse CT scans showing a HCC of 2 cm in the right lobe treated with multisession PEI. (**a**, **b**) Before treatment the tumor shows hypervascularity during the arterial phase and washout in the portal phase. (**c**, **d**) The arterial and portal phase CT scans the day after treatment show a completely necrotic lesion because of the absence of enhancement. Very small bubbles of gas due to recent necrosis are detectable inside the treated area.

markers is used. They are the same as those adopted during initial staging and controls. Since there are many investigations and some of them are comparable, we prefer to routinely use only contrast-enhanced US (CEUS) (with SonoVue, Bracco, Milan, Italy) and spiral multislice CT (Fig. 2) with the triphasic technique (4–5 ml/s, 30, 70, and 120 s after the injection of contrast medium). Other imaging techniques (angiography, MR, PET) or biopsy is performed only in rare cases, if there is a doubt whether the response is partial or complete. If the areas of viable tissue are very small, beyond the

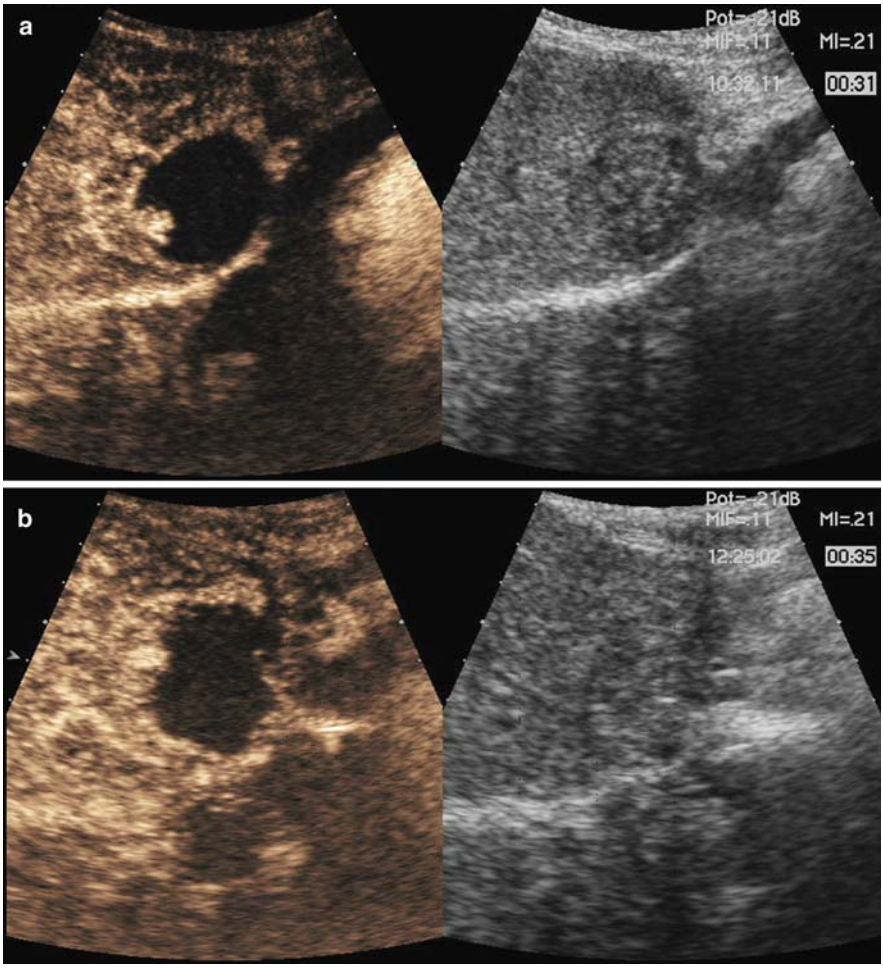

Fig. 3. Contrast-enhanced US scans showing a HCC nodule treated with multisession PEI. (**a**) Vital hypervascularized tissue remains present after the first session. (**b**) After the second treatment, targeted using contrast-enhanced US as guidance, the lesion is completely treated.

present powers of resolution, they will obviously not be recognizable on the images at the end of the treatment. However, they will be easily identified at follow-up if they are evidenced as zones of enhancement at CT or CEUS. The response is considered complete when CT and CEUS scans show the total disappearance of enhancement within the neoplastic tissue and when the same picture is confirmed at scans performed at successive controls.

The absence of enhancement means the absence of blood flow due to necrotic and fibrotic modifications. Even with such characteristics, the necrotic area does not disappear and remains visible in place of the tumor even if reduced in size to different extents.

CEUS is particularly useful *(14–15)* during multisession treatment as it permits to evaluate before each session if there is persistence of any viable area. The following instillation of ethanol can therefore be selectively performed in the tumoral tissue (Fig. 3).

As tumor markers, we use α-fetoprotein (AFP) and des-γ-carboxy-prothrombin (DCP), which are often complementary. Nevertheless, their assay is useful only if they were abnormal before treatment. When the imaging techniques show a complete response not followed by normalization of AFP or DCP levels, it means that neoplastic tissue not detected or not yet detectable is growing elsewhere. Moreover, an increase in levels during follow-up always suggests a local recurrence or the appearance of new lesions. The control with CEUS and/or CT is carried out according to the procedure used. If the multisession procedure is performed, the control is made when the treatment is presumed to be complete. If the "single-session" procedure is performed, the control is made the day after treatment. After that these imaging examinations and serum assay of tumor markers are performed every 4–6 months.

4. COMPLICATIONS

Mortality related to conventional treatment is negligible, because only few anecdotal cases were reported in thousands of patients treated. In a review study with 1066 patients treated in 8118 sessions, one death (0.09%) occurred *(16)*. Major complications are rare, ranging from 1.3 to 2.4%, and usually treated conservatively (intraperitoneal hemorrhage, cholangitis, jaundice secondary to injury of main bile ducts, liver abscess, hemobilia, arterioportal shunt, shock, and segmental hepatic infarction).

With the "single-session" technique, where larger volumes of ethanol are administered, the mortality (0.9%) and the complication rate increase (4.5%), and other major complications can occur (transient worsening of portal hypertension with risk of hemorrhage from esophageal varices, liver decompensation, transient alcohol intoxication) *(9)*.

A particular and late type of complication is seeding, which may occur despite the use of small needles and injecting alcohol down the track. In a recent study *(17)* with a large cohort of patients, the authors registered five cases of seeding out of 270 patients (1.8%).

A review article *(18)* evaluated all the cases of seeding following PEI without prior biopsy reported between January 1983 and February 2007. A total of 16 papers describing 26 cases of seeding were found. The most common site of seeding was intraperitoneal and the median time from PEI to detecting seeding was 6 months.

5. RESULTS

5.1. Survival Studies

Numerous long-term survival curves have been published. The more important studies in terms of quality and quantity were conducted in Italy and in Japan [7-8-11-12]. Their 5-year survival, in patients with single HCC $\leq$5 cm or with $\leq$3 nodules $\leq$3 cm, ranged from 43 to 63%. Most recently Ebara *(17)* reported an overall 3- and 5-year survival rates of 81.6% and 60.3%, respectively. The rates were higher (87.3% at 3 years and 78.3% at 5 years) in Child A patients with a solitary tumor $\leq$2 cm in diameter.

Main pretreatment factors influencing survival are liver function, tumoral markers (AFP, DCP) level, number, and size of tumors. A post-treatment prognostic factor is the complete response to PAT *(19)*. The main cause of death in Child A patients was progression of neoplastic disease due mainly to the appearance of new lesions, while in Child C patients the cause of death was hepatic insufficiency, questioning the useless of treatment in these patients.

The incidence of appearance of new lesions at 5 years ranged from 64 to 87%, i.e., the same rates showed after surgery. The incidence of local recurrences ranged from 4 to 17%, usually due to the tumor.

Following these results, the European and the American Associations for the Study of the Liver included PEI among the treatments considered effective for early-stage disease *(20)*.

5.2. Comparison to Other Therapies

In all the randomized controlled trials (RCTs), RF showed better local efficacy and required fewer treatment sessions compared to PEI, but PEI presented a minor rate of adverse events *(21, 22)*. In particular, in tumor <3 cm in size, RF obtained a complete ablation in nearly the totality of cases, while PEI obtained approximately 10% less. Successively, RF was compared to PEI for long-term results. In all the RCTs, RF was superior

to PEI with respect to local recurrence, overall survival, and cancer-free survival *(23–25)*. For explaining the difference regarding these parameters, it is important to remember that also at the earliest stages *(26)* different degrees of tissue differentiation are possible. Histopathologic studies have revealed that, while nodules measuring 1.5 cm or less (considered the early stage for pathologists) are uniformly well differentiated, those between 1.5 and 2.0 cm in diameter often contain zones of less-differentiated tissue with more intense proliferative activity (considered the small advanced stage for pathologists) *(27–29)*. The less-differentiated areas give rise to portal microinvasion in 10% of the cases and to microsatellites in 3% of the cases, usually within 1.0 cm of the main tumor *(28–31)*. Better long-term results of RF are due to the fact that thermoablation in most cases of early stages is able to obtain a 0.5–1.0 cm safety margin around the tumor, reducing the appearance of possible microsatellites during the follow-up. RF resulted superior to PEI also in tumors of medium and large size *(32)*.

Recently an RCT on 184 patients with HCC $\leq$3 cm found that RF was superior to PEI and PAI with respect to local recurrence, overall survival, and cancer-free survival rates, even if RF caused more major complications (4.8% vs 0%). No statistically significant difference was reported between PEI and PAI *(33)*.

Some retrospective studies comparing PEI and hepatic resection (HR) showed 5-year survival rates broadly equivalent, with an approximate rate of 50% for both *(34–37)*. These data were recently confirmed by the only RCT which compared patients with one or two nodules $\leq$3 cm in size, which did not find any statistical difference for recurrence rate and survival *(38)*.

5.3. Combined Therapies

Combined therapy with PEI and RF for large HCC has been proposed demonstrating that the two techniques cause a synergistic necrotizing effect, with coagulation volumes larger than those usually obtained with PEI or RFA alone *(39*, *40)*. Recently the combination of repeated single-session PEI and transarterial chemoembolization (TACE) has been compared to repeated single-session PEI in patients with nonresectable HCC *(41)*. The combination of TACE and PEI was associated with a longer survival (1-, 3-, and 5-year survival: 90, 52, and 43%, respectively) compared to PEI treatment alone (1-, 3-, and 5-year survival: 65, 50, and 37%, respectively).

6. CONCLUSIONS AND CURRENT INDICATIONS

HCC usually coexists with an underlying hepatic chronic disease. According to the stage, one disease will prevail over the other. For such reason, therapies should not worsen liver function. HCC is an organ

pathology, so the first nodule detected is only a prelude to others. A study on resected patients demonstrated that multicentricity is already present in 50% of early stages and that 93% of patients with single minute HCC presented other nodules within 5 years *(42)*. Being multicentric over time, HCC needs multistep treatments.

Therefore HR (or PATs) can offer a palliative cure, achieving only a local control of the disease. In fact, according to a Japanese nationwide survey, only 1.6% of all resected patients presenting intrahepatic recurrence were re-resected *(43)*.

Although it is understood that HR assures the highest possibility to completely ablate the tumor and the possible satellites, different comparative studies based on historical results *(35–37)* and the recent RCTs comparing HR and PATs demonstrated roughly equivalent results *(34*, *38*, *44*, *45)*. The explanation is probably due to a balance between advantages and disadvantages of the two therapies, the most important advantages of PATs being repeatability, no loss or damage of non-neoplastic tissue, and lower complication rates. Moreover, the overall results of both therapies were hampered and flattened by an incorrect selection of the patients, part of them being treated even though they had adverse prognostic factors for that specific treatment. For instance, the Liver Unit of Barcelona reported the usual, i.e., the mean rate reported by most studies, 5-year overall survival rate of around 50% after HR *(46)*. However, when the patients were divided according to two simple adverse prognostic factors, i.e., portal hypertension and abnormal bilirubin, a rate of 74% was obtained (the best so far reported) in patients with normal values and a rate of only 25% in the worst candidates. The fact that the survival of this second group of patients was comparable with recently reported survival rates from two series of untreated patients (20 and 16%, respectively), even though with a more adverse profile *(47*, *48)*, questions the indication for surgery in such patients that are probably more eligible for PATs.

These considerations suggest that the best strategy has to be tailored according to the individual presentation of the disease. In single operable nodule <3 cm, there is no clear evidence to establish the best treatment. Accordingly, each referral center follows a personal algorithm for such borderline patients. Currently, RF is becoming the gold standard for nodules <2 cm *(49)*, while for nodules between 2 and 3 cm the choice is reached according to individual factors.

As RF is actually considered the gold standard ablation technique, the current place of PEI has to be determined. Of course where RF is not available PEI remains a valid treatment for HCC, especially for health-care systems with limited economical resources as studies related to the total cost of treatment reported an average of only 700–1000 $ for PEI *(8*, *50)*.

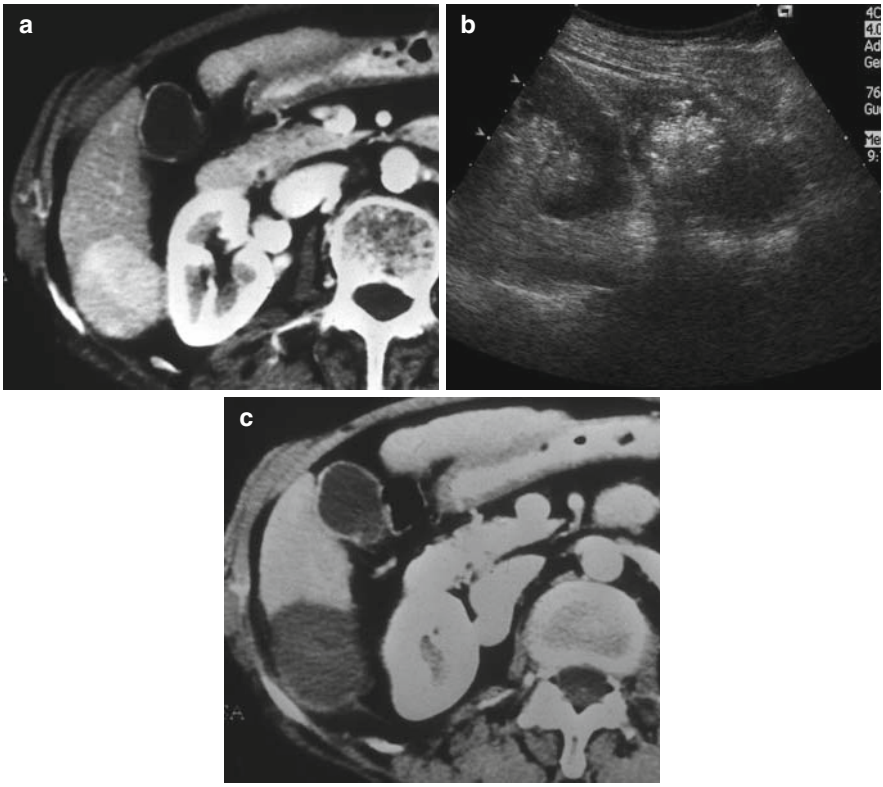

Fig. 4. Transverse CT and US scans showing a HCC of 4.2 cm located in segment VI, close to the bowel, treated with single-session PEI because of its at-risk location. (**a**) At the baseline the lesion appears well vascularized at arterial phase CT scan. (**b**) US scan at the end of the procedure shows the hyperechoic zone of ethanol filling the tumor. (**c**) At the arterial phase CT scan 1 month after treatment no enhancement is visible within the tumor.

Moreover in all those cases in which RF is considered to be at risk for complications, PEI is a valid alternative, i.e., in case of lesions adjacent to main biliary ducts (because of the risk of stenosis) or to intestinal loops (Fig. 4) (above all when fibrotic adhesions between the hepatic capsule and the intestinal wall are suspected, because of the risk of perforation) *(51)*. Combined therapies have been also proposed for these kinds of lesions *(52*, *53)*. PEI is also useful to treat lesions close to large vessels, as it is not affected by the so-called sink effect. PEI remains a good indication to treat segmental portal thrombosis.

In our department we consider PEI and RF, and also selective TACE, complementary, and use them according to the presentation of the disease, i.e., size, number, location, and presence of satellites or portal thrombosis. A

multifocal HCC can be treated with only one or with all the techniques, during a single hospital stay or over the years. Our longest survivor, currently free of disease, was initially treated 19 years ago with PEI and when new lesions appeared during follow-up, he was treated with RF, selective TACE, and again PEI. Otherwise, the same lesion can also be treated with the combination of different techniques when the first has resulted unsatisfactory.

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16 Radiofrequency Ablation of Hepatocellular Carcinoma

Kevin Tri Nguyen, MD, PhD
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CONTENTS

ABSTRACT

We provide a historical perspective and review on the current status of radiofrequency ablation (RFA) in the treatment of hepatocellular carcinoma (HCC). Currently, HCC accounts for 85–90% of primary liver cancer, which is the sixth most common cancer worldwide. For qualified candidates, liver

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transplantation and surgical resection provide the only chance for cure. For the subset of patients who do not qualify for transplantation or surgical resection, RFA provides a locoregional alternative to the treatment of HCC. RFA uses a radiofrequency energy-generated heat to destroy biological tissues and has been used to destroy unresectable malignant liver tumors since the early 1990s. Currently, there are three FDA-approved RFA systems: the RITA Starburst, LeVeen, and Cool-tip RFA systems. Liver transplantation and surgical resection provide the best change for cure and long-term survival. RFA should be reserved for those patients who are deemed unresectable, either based on tumor size, number, location, major blood vessel invasion, inadequate hepatic reserve, or significant comorbidities. Absolute contraindications to RFA include the presence of extrahepatic disease, life expectancy less than 6 months, altered mental status, active infection, and tumor abutting a major hepatic duct. Relative contraindications include lesions greater than 5 cm (especially in a cirrhotic liver), more than four lesions, severe pulmonary or cardiac disease, and refractory coagulopathy. Compared to surgical resection, RFA was associated with a higher local recurrence and a shorter overall and disease-free survival. However, RFA was superior to percutaneous ethanol injection (PEI) with more complete response while requiring less treatment sessions and significantly improved local recurrence-free survival, overall survival, and disease-free survival. The addition of transarterial chemoembolization (TACE) significantly improved survival compared to RFA alone or TACE alone. Finally, RFA also provides a safe bridge to transplant with significant complete necrosis observed with tumors <3 cm and nonperivascular tumors. In summary, surgical resection remains the gold standard in those patients who do not meet transplant criteria. If resection is not an option or if the patient chooses a less invasive approach, then RFA is a viable option and may be comparable to resection in select patients. RFA appears to be superior to PEI for the percutaneous treatment of unresectable HCC and should the standard percutaneous technique. For patients who meet transplant criteria, prolonged waiting may result in tumor growth with progression to vascular invasion, which is highly associated with post-transplant recurrence; thus, RFA provides a safe, minimally invasive bridge to transplantation.

Key Words: Hepatocellular carcinoma; radiofrequency ablation

1. INTRODUCTION

Primary liver cancer is a major concern globally. It is the sixth most common cancer worldwide (behind lung, breast, colorectal, stomach, and prostate cancers) with an estimated 626,000 new cases diagnosed annually

as of 2002, but it is the third most common cause of death from cancer (behind lung and stomach cancers) *(1)*. Hepatocellular carcinoma (HCC) accounts for 85–90% of the primary liver cancers. Major risk factors include infections from hepatitis B or C virus. Chronic HBV carriers have a 5–15-fold increased risk of HCC, while HCV-infected patients have 17-fold increased risk of HCC compared to the general population *(2)*. Most HCC cases (>80%) occur in the sub-Saharan Africa or Eastern Asia, with the incidence as high as 28–49/100,000 males per year and 12–15/100,000 females per year, while the incidence in the United States is 3.3/100,000 persons per year. HCC develops in background of chronic liver disease and cirrhosis in approximately 70–90% patients with HCC. The annual conversion rate of someone with cirrhosis to develop HCC is 0.26–0.6% in HBV carriers and 1–3% in HCV carriers after 25–30 years of chronic infection *(2)*.

Given the high incidence of HCC in the background of chronic liver disease and cirrhosis, any surgical therapy must consider not only the cancer but also the underlying liver function and reserve. It is often the degree of liver dysfunction/reserve that will determine the optimal treatment. At the present time, liver transplantation or surgical resection offers the only chance for cure in the small subset of eligible patients. Five-year survival after transplantation ranges from 61 to 75% *(3)*, while 5-year survival after surgical resection ranges from 31 to 93%, with the 93% 5-year survival rate seen in very early HCC detected with screening seen in Japan *(3–5)*. Surgical resectability for HCC ranges from 10 to 35% in patients *(6–9)*. Contraindications to resection include extrahepatic involvement, multifocal, bilobar disease, inadequate hepatic reserve, or overall poor clinical condition of the patient.

Many treatment options exist in an attempt to palliate patients with unresectable HCC. They include intra-arterial chemotherapy, ethanol injection, chemoembolization, cryotherapy, radiofrequency ablation, and systemic chemotherapy. A historical perspective and the current status of radiofrequency ablation (RFA) in the treatment of HCC will be reviewed in this chapter.

2. RFA HISTORICAL BACKGROUND

Hippocrates (460–370 BC) once said, "...diseases which medicines do not cure, iron cures; those which iron cannot cure, fire cures; and those which fire cannot cure, are to be reckoned wholly incurable." *(10)* The use of heat energy to heal has been long known in history. The Greeks used heated stones for medicinal purposes and the ancient Hindu heated metal bars to stop bleeding *(11)*. Antoine Henri Becquerel, a French physi-

cist and the 1903 Nobel laureate in Physics for the discovery of radioactivity, was the first to demonstrate electrocautery when he heated a needle with electricity to cauterize wounds to stop bleeding. However, the breakthrough in the use of electrocautery in surgery occurred in 1891 when Jacques-Arsène d'Arsonval, a French physician and physicist, discovered that radiofrequency (RF) waves at an alternating electrical current greater than 10 kHz could pass through living tissue without pain or neuromuscular excitation *(6*, *12*, *13)*. The resistance of the tissue to the rapidly alternating current would generate heat. The first clinical use of this technology was in 1908 by Edwin Beer who used RF coagulation to destroy urinary bladder tumors through a cystoscope *(14)*. Harvey Cushing and William T. Bovie later applied RF ablation to intracranial tumors *(15)*. W. Lounsberry in 1961 studied the histological changes of the liver after RFA in animal models *(16)*. He found that RF caused local tissue destruction with uniform necrosis. There was a demarcation line between normal cells and necrotic cells. Cooling from the circulation prevented thrombosis of adjacent blood vessels.

In the early 1990s, two independent groups of investigators proposed that RFA can be an effective method for destroying unresectable malignant liver tumors *(17*, *18)*. Both groups found that RF lesions had a well-demarcated area of necrosis without any viable tumor cells present. Subsequent animal and human trials have suggested that RF is safe and effective in the treatment of liver tumors *(19–21)*.

3. MECHANISM OF ABLATION

An understanding of the physics of radiofrequency is essential to achieve an effective ablation. Radiofrequency thermal ablation is the use of radiofrequency energy-generated heat to destroy biological tissue. The thermal injury is due to frictional heat generated by the ionic agitation of particles within tissue following the application of alternating current *(22)*. The electrode transmits alternating current within the radiofrequency range (200–1,200 MHz), resulting in frictional heat in the surrounding tissue that causes cellular destruction and tissue necrosis *(23)*. The heat generated around the electrode is rapidly dissipated within a short distance from the electrode. To increase the volume of ablated tissue, the shape, size, and position of the electrode is altered *(24)*. If the current generated is too high or applied too rapidly, the ablated area will be irregular or small. If the temperature of the tissue surrounding the electrode reaches 110°C, rapid desiccation will occur, causing tissue adherence to the electrode, which then acts as an insulator and impedes further flow of the current. The optimal temperature for coagulation of liver tissue to occur is 80–100°C with a minimum of 50°C *(25*, *26)*. Tumor death begins to occur at 60°C with a minimum of 1 cm necrosis occurring at 71°C *(24*, *27*, *28)*.

4. RFA EQUIPMENT

Three different RFA systems are currently available with FDA approval for biomedical devices. In January 2007, AngioDynamics (Queensbury, NY) acquired RITA Medical Systems, which offers a number of radiofrequency ablation systems. The RITA® Model 1500X RF generator has a maximal 250 W power output operating at 460 kHz frequency. The generator is started at 25 W and slowly increased over a few minutes. Once the temperature is around 100°C, the electrodes are fully deployed. The temperature is maintained constant by adjusting the watts applied over a certain time interval. The Starburst ablation device has nine electrodes, a live trocar tip, and provides real-time temperature feedback from five independent thermocouples within the array. A number of models have emerged. Please see the AngioDynamics website for further information (www.angiodynamics.com).

RadioTherapeutics Corporation (Sunnyvale, CA) was acquired by Boston Scientific Corporation (www.bostonscientific.com). They offer a family of LeVeen RFA electrodes capable of achieving ablations 2–5 cm in size with a unique, patented umbrella-shaped array design that can be used for percutaneous, laparoscopic, or open radiofrequency ablation. The LeVeen Needle Electrodes are currently available with array diameter ranging from 2.0 to 5.0 cm (2.0, 3.0, 3.5, 4.0, 5.0 cm) and cannula length options of 12, 15, or 25 cm (the longest for laparoscopic RFA). The RF 2000 and RF 3000 generators used with the Lee Veen needle electrodes produce maximum powers of 100 and 200 W, respectively. The main difference between the RF 2000/3000 LeVeen system and RITA StarBurst systems is that RF 2000/3000 system uses tissue impedance as feedback monitoring, while RITA system relies on temperature. The power is gradually increased over a 10–15-min period until the impedance rises over 200 Ω, achieving "roll-off". A second phase of thermal ablation is used for each deployment.

Radionics (Burlington, VT) was acquired by Covidien, a division of Tyco Healthcare Group LP, and is the third company offering a RF ablation system (www.covidien.com). Their design is the Cool-tip 17-gauge hollow needle RFA electrode that can record the temperature of the surrounding tissue (www.cool-tiprf.com). The power generator is a 200-W box using 480 kHz alternating current and can display temperature as well as tissue impedance. The feedback algorithm continually monitors tissue impedance, automatically adjusting output to maximize energy delivery. During the ablation, internal channels allow chilled water to perfuse the needle, cooling adjacent tissue. The cooled needle prevents charring around the electrode tip and keeps resistance low to produce a larger ablation zone *(6*, *27*, *29*, *30)*. The cool-tip needles are available as a single electrode achieving 2–4 cm ablations or a cluster of three single electrodes in a triangular pattern to achieve larger ablations. Similar to the other companies, the electrodes vary in length from 10 to 25 cm.

Lin et al. conducted a prospective comparison of the RF 2000 with LeVeen electrode, RF 3000 with LeVeen electrode, RITA with StarBurst electrode, and Cool-tip radiofrequency ablation systems for the ablation of HCC *(31)*. There was no significant difference in the rate of complete necrosis between the four groups (91.1–96.8%). The number of sessions of RFA to achieve complete tumor necrosis was higher in the RF 2000 group (1.4 ± 0.6) compared to the other groups ($p < 0.05$). The average time to complete ablation was shortest with the RF 3000 group (16.6 min) compared to the other groups (31.7 min in the RF 2000 group; 28.3 min in the RITA group; 27.1 min in the Cool-tip group) ($p < 0.005$). There was no significant difference in the rate of local tumor progression at 2 years (8–12%) and no significant difference in the development of new HCC recurrence (24–32%). There was no significant difference in overall (73–78%) and disease-free survival (54–56%) at 2 years between the four groups.

5. EVALUATION AND PATIENT SELECTION

Metastatic colorectal cancer and HCC are the major hepatic tumors treated by RFA. Surgical resection, however, remains the "gold standard" in any patient harboring a HCC or a metastatic tumor that is amenable to resection. Unfortunately, concurrent cirrhosis and inadequate predicted functional liver reserve after resection often limit the ability of the patient to tolerate a major resection. These patients should be considered for liver transplantation if they meet Milan criteria (see accompanying chapter). RFA should be reserved for those patients who are deemed unresectable either based on tumor size, number, location, major blood vessel invasion, inadequate hepatic reserve, or significant comorbidities. RFA has also been used as a bridge in patients with cirrhosis that develop a small HCC while awaiting a liver transplant *(32, 33)*. RFA can also be used to expand the operative indication in a subset of patients that have a resectable lesion in one lobe and a deep lesion in the contralateral lobe. Patients at risk for HCC are screened using α-fetoprotein (AFP) and des-γ-carboxyprothrombin serum tumor markers, as well as radiographic imaging with a triphasic CT scan or contrast MRI.

Absolute contraindications to RFA include the presence of extrahepatic disease, life expectancy less than 6 months, altered mental status, active infection, and tumor abutting a major hepatic duct (Table 1). Although there is no uniform agreement in the literature, relative contraindications include lesions greater than 5 cm (especially in a cirrhotic liver), more than four lesions, severe pulmonary or cardiac disease, and refractory coagulopathy. Tumors greater than 5 cm require overlapping fields with the current electrode technology and are associated with increased risk of abscess formation *(34)*.

Table 1
Contraindications for RFA

Absolute contraindications
1. Extrahepatic disease
2. Life expectancy less than 6 months
3. Altered mental status
4. Active infection
5. Tumor abutting a major hepatic duct
Relative contraindications
1. Lesions >5 cm especially in cirrhotics
2. More than four lesions
3. Severe pulmonary or cardiac disease
4. Refractory coagulopathy

6. PROCEDURE

With each RFA procedure, the goal is to thermally ablate the entire lesion and a 1-cm rim of normal liver at the tumor margin *(35)*. The route of RFA electrode delivery can be either percutaneous, laparoscopic, or open. The percutaneous approach is performed either in the radiology suite or in the operating room. Due to the pain associated with the procedure, sedation and intravenous narcotics are required for the awake, percutaneous approach. For laparoscopic or open RFA cases, the procedure is performed under general anesthesia. Minimally invasive approaches (percutaneous or laparoscopic) are preferable to the patient. Several factors must be taken into consideration in deciding the best strategy for each patient. These include the number of lesions, size, and location. For example, a lesion extending to the liver capsule in the left lobe or the caudal side of the right lobe may actually be in close proximity to the stomach or the colon. Percutaneous targeting risks thermal injury to these organs and is better handled by the laparoscopic or open approach. A history of multiple prior abdominal operations with adhesions may preclude adequate laparoscopy and require an open approach. A lesion high in the dome of the right lobe can also be challenging. By percutaneous route, the electrode must traverse the lung and the diaphragm, risking pneumothorax or bleeding. By laparoscopic approach, the high lesions can be difficult to reach.

At the University of Pittsburgh, the RFA equipment of choice is the RadioTherapeutics/LeVeen Needle Electrode system. To be eligible for a percutaneous approach, we prefer a solitary, intrahepatic tumor smaller than 3 cm and readily visualized on ultrasound. When there are multiple lesions or the tumor is not safely accessible by percutaneous route, the laparo-

scopic approach is preferred unless the patient is undergoing another procedure such as resection that requires a laparotomy. If the procedure is being performed percutaneously or laparoscopically, a sheathed needle is used to puncture the skin. The needle is removed leaving the sheath for passage of the RFA electrode. This minimizes the theoretical risk of tumor seeding along the needle track in the abdominal wall *(36)*.

Regardless of the approach, ultrasound guidance is used to place the needle electrode into the tumor. It requires careful positioning to avoid leaving any viable tumor behind. If the lesion is too large to be completely targeted with one deployment, then the deep margin is ablated first, followed by electrode withdrawal to get the superficial margin. Once the ablation is initiated, gas in the tissue obscures visualization beyond the deep margin. During the procedure, the area of ablation develops a zone of increased echogenicity and microbubbles *(37)*. There is conflicting evidence as to whether ultrasound immediately post-ablation can assess the adequacy of the treatment. Some studies have shown that this does not give an accurate assessment of the tumor margins *(38*, *39)*. Cioni et al. evaluated the use of a contrast-enhanced harmonic power Doppler ultrasound vs biphasic helical CT scan in evaluating post-ablation lesions in 50 patients with HCC *(40)*. Using a microbubble contrast agent, they found that the Doppler ultrasound had similar results in evaluating the thermal zone of destruction compared to CT scan. During laparoscopy or laparotomy, vascular inflow occlusion with a Pringle maneuver can be performed to facilitate achieving a larger zone of ablation by decreasing the heat sink of the adjacent blood vessels *(41*, *42)*. For follow-up, it is our practice to obtain CT scans at 3 and 6 months after the RFA, although some groups recommend follow-up scan as early as 1 month post-RFA *(43*, *44)*. Depending on the level of concern, we then obtain subsequent scans at 6 months (or more frequently) for 2 years. Rising tumor markers or scans showing contrast enhancement at the periphery of the ablation zone suggest recurrence.

7. RFA VS HEPATECTOMY FOR HCC

Surgical resection is the gold standard in those patients who do not meet transplant criteria *(45–47)*. However, the resection option is suitable for only 10–35% of patients presenting with HCC mainly due to extrahepatic disease, multifocal, bilobar disease, inadequate hepatic reserve, or overall poor clinical condition of the patient *(7–9*, *36*, *48–53)*. If resection is not an option or if the patient chooses a less invasive approach, then ablation is a viable option and may be comparable to resection in select patients. A number of studies have compared RFA with hepatectomy for HCC.

Guglielmi et al. conducted a retrospective review comparing 91 surgical resections vs 109 RFA for HCC *(54)*. Overall survival and disease-free survival were significantly longer in patients who underwent surgical resection

vs those who were treated with RFA (57 months vs 28 months overall survival, $p = 0.01$; 36 months vs 16 months disease-free survival, $p = 0.001$). However, in a select group of patients who were Child-Pugh class B, who had multiple HCC, or who had HCC lesions smaller than 3 cm, there was no difference in overall and disease-free survival.

Wakai et al. retrospectively reviewed 149 patients with HCC $\leq$4 cm who underwent surgical resection ($n = 85$) vs percutaneous ablation ($n = 64$), which included RFA ($n = 21$), percutaneous ethanol injection ($n = 37$), and microwave coagulation ($n = 6$) *(55)*. Local recurrence was significantly less frequent following hepatectomy ($p < 0.0001$). The incidence of local recurrence reached a plateau of 28% at 20 months after percutaneous ablation, while a plateau of 3% at 22 months after hepatectomy. Survival was longer after hepatectomy with a mean survival time of 122 months (10-year survival rate of 53%) vs 66 months (10-year survival rate of 31%) after percutaneous ablation ($p = 0.0123$). When the patients were subdivided by tumor size $\leq$2 cm or >2 cm, improved and long-term survival from hepatectomy was observed only in tumors >2 cm ($p = 0.0001$). For tumors $\leq$2 cm, no local recurrence was observed after hepatectomy during the follow-up period, while local recurrence plateaued at 12% at 15 months after percutaneous ablation, although this was not statistically significant ($p = 0.0881$). In addition, among patients with tumors $\leq$2 cm, mean survival time was 122 months (10-year survival rate of 58%) after hepatectomy, while mean survival rate was 76 months (10-year survival rate of 45%) after percutaneous ablation, but again, not statistically significant ($p = 0.0813$).

Vivarelli et al. compared 79 cirrhotics with HCC who underwent RFA vs 79 cirrhotics with HCC who underwent surgical resection *(56)*. No patients received neoadjuvant or adjuvant therapy before or after the main procedure. Overall survival at 1 year and 3 years was significantly improved after hepatic resection for HCC (83 and 65%) compared to the 1- and 3-year survival of patients who underwent RFA for HCC (78 and 33%) ($p = 0.002$). In addition, disease-free survival at 1 year and 3 years was also significantly better after hepatic resection for HCC (79 and 50%) compared to those who were treated with RFA for HCC (60 and 20%) ($p = 0.001$). This surgical advantage was most evident in patients with Child-Pugh class A and single tumors >3 cm ($p = 0.001$). For patients with tumors $\leq$3 cm, there was a trend toward better outcomes after surgical resection vs that of RFA (overall 3-year survival 79% vs 50%, respectively; 3-year disease-free survival 67% vs 34%, respectively), although this did not reach statistical significance due to small sample size.

In a select group of patients with well-preserved liver function and a single HCC <4 cm, Hong et al. found that there was no significant difference between surgical resection and RFA in the incidence of remote recurrence, overall survival, and disease-free survival at 1 year and 3 years *(57)*. Local recurrence, however, was significantly higher in those patients

who underwent RFA compared to patients who underwent surgical resection ($p = 0.005$), but this did not affect overall or disease-free survival as the local recurrences were treated with repeat RFA or with transarterial chemoembolization (TACE).

8. RFA VS TACE VS COMBINED RFA+TACE FOR HCC

Recently, Cheng et al. reported the first randomized clinical trial involving RFA for HCC in 291 Chinese patients with three or fewer HCC tumors ranging in size from 3 to 7.5 cm *(58)*. Patients were randomly assigned to treatment arms of RFA alone ($n = 100$), TACE alone ($n = 95$), or combined TACE+RFA($n = 96$). With a median follow-up of 28.5 months, median survival was 22 months in the RFA group, 24 months in the TACE group, and 37 months in the TACE+RFA group. Patients treated with TACE+RFA had better overall survival than those treated with TACE alone ($p < 0.001$) or RFA alone ($p < 0.001$).

9. RFA VS PEI FOR HCC

Percutaneous ethanol injection (PEI) was introduced by Livraghi et al. in 1986 *(59)* and was considered the standard percutaneous technique by the European Association for the Study of the Liver (EASL) for the treatment modality for small, unresectable HCC in 2000 until further studies could compare PEI to other percutaneous techniques *(47)*. Since then, a number of randomized, controlled trials have compared RFA to PEI for the treatment of HCC.

Lencioni et al. conducted a prospective, randomized study comparing 52 patients who underwent RFA vs 50 patients who underwent PEI for either a single HCC $\leqq$5 cm or $\leqq$3 HCCs that are each $\leqq$3 cm *(60)*. Complete tumor response was successful in 91% of HCCs treated with RFA with an average of 1.1 treatment sessions, but only in 82% of HCCs treated with PEI, which required an average of 5.4 treatment sessions. At a mean follow-up of approximately 22 months, there was a trend toward survival in patients undergoing RFA vs PEI at 1-year (100% vs 96%) and 2-year (98% vs 88%) follow-up, although it was not statistically significant ($p = 0.138$). However, local recurrence-free survival was significantly better in patients who underwent RFA than in patients who underwent PEI for HCC at 1-year (98% vs 83%) and 2-year (96% vs 62%) follow-up ($p = 0.002$). This study suggests that RFA is superior to PEI in the treatment of patients with small HCCs who do not qualify for either transplantation or resection.

Lin et al. randomized 187 patients with HCC $\leqq$3 cm in diameter to either percutaneous radiofrequency ablation ($n = 62$), percutaneous ethanol injection ($n = 62$), or percutaneous acetic acid injection (PAI) ($n = 63$) *(61)*. They

found that RFA was superior to PEI and PAI in terms of local recurrence, overall survival, and disease-free survival. Specifically, 3-year local recurrence rates were 14% in the RFA group, 34% in the PEI group, and 31% in the PAI group (RFA vs PEI, $p = 0.012$; RFA vs PAI, $p = 0.017$). Three-year overall survival rates were 74% in the RFA group, 51% in the PEI group, and 53% in the PAI group (RFA vs PEI, $p = 0.031$; RFA vs PAI, $p = 0.038$). Three-year disease-free survival rates were 43% in the RFA group, 21% in the PEI group, and 23% in the PAI group (RFA vs PEI, $p = 0.038$; RFA vs PAI, $p = 0.041$).

Shiina et al. conducted a randomized, controlled trial comparing radiofrequency ablation to percutaneous ethanol injection in 232 patients with HCC who had less than or equal to three lesions, each $\leqq$3 cm in diameter *(62)*. RFA, compared to PEI, required less number of treatment sessions (2.1 times vs 6.4 times, $p < 0.0001$) and shorter hospital stay (10.8 days vs 26.1 days, $p < 0.0001$). RFA was associated with a 46% smaller risk of death ($p = 0.02$), a 43% smaller risk of overall recurrence ($p = 0.0009$), and an 88% smaller risk of local recurrence ($p = 0.006$). Four-year survival after RFA was 74% vs 57% after PEI.

Overall, radiofrequency ablation appears to be superior to percutaneous ethanol injection for percutaneous treatment of unresectable HCC and should be the standard percutaneous technique.

10. RFA PRIOR TO LIVER TRANSPLANTATION

The majority of patients with HCC do not meet transplant criteria. Of those few who do qualify for liver transplantation, the wait time may be months to years. The median time from listing to actual liver transplantation for patients in the United States with a T2 HCC exception holders was 48 days in 2006 *(63)*. No randomized controlled trial has shown that RFA reduces the rate of dropout, helps down-stage HCC lesions, or improves survival *(63, 64)*. However, if waiting is prolonged, tumor growth during the waiting period may progress to vascular invasion, which is highly associated with post-transplant recurrence. Percutaneous RFA provides a safe, minimally invasive bridge to transplantation.

Pompili et al. retrospectively analyzed 40 patients who underwent percutaneous treatment of 46 HCC lesions prior to transplantation *(65)*. The lesions were treated either by RFA (65.2%), PEI (28.3%), or the combination of RFA+PEI (6.5%). Mean waiting time for OLT was 9.5 months. Examination of the explanted liver revealed complete necrosis in 46.7% of lesions treated by RFA and 23.1% treated by PEI. Complete tumor necrosis independent of treatment was possible in 53.1% for lesions $\leqq$3 cm and only 14.3% for lesions >3 cm ($p = 0.033$), but complete necrosis increased

to 61.9% in lesions ≦3 cm treated by RFA. For patients with HCC treated prior to OLT, RFA appears to be the best percutaneous treatment modality, especially for lesions ≦3 cm.

Mazzaferro et al. conducted a prospective study on 50 cirrhotic patients with 60 HCC who underwent RFA as a bridge to liver transplantation *(66)*. The mean interval from RFA to OLT was 9.5 months. Complete tumor necrosis was 55%, but increased to 63% for HCC ≦3 cm ($p = 0.007$). Three years after liver transplantation, patient/graft survival was 83%.

Lu et al. reviewed the outcome of 52 patients with 87 HCC lesions treated by percutaneous RFA prior to orthotopic liver transplantation *(67)*. Mean waiting time was 12.7 months, with a dropout of 5.8% due to tumor progression. Complete tumor necrosis was observed in 85.1% of patients by post-ablation imaging. On histological examination, complete necrosis was observed in 83% of tumors ≦3 cm in size, compared to only 50% of tumors >3 cm in size ($p = 0.05$), and in 88% of nonperivascular tumors vs 47% of perivascular tumors ($p = 0.009$). Liver transplantation was completed in 78.8% of patients. Three-year survival was 76% with no recurrences.

In contrast to the three previous mentioned studies, one study from the United States found no benefit in pretransplant locoregional therapy in the Model for End-Stage Liver Disease (MELD) era. Porrett et al. retrospectively compared 31 treated and 33 untreated controls *(68)*. After 36 months of follow-up, they found no difference in overall survival (84% vs 91%), disease-free survival (74% vs 85%), tumor recurrence (23% vs 12%), or mortality from tumor recurrence (57% vs 25%) ($p > 0.1$). Mean time transplant after MELD assignment was 54 days in the treated patients and 119 days in the untreated patients ($p = 0.05$). In addition, for the treated group, complete tumor necrosis was demonstrated on histological examination in only 20% of explanted livers, which is less than that demonstrated by the studies outside of the United States.

11. RECURRENCE, MORALITY, AND LONG-TERM SURVIVAL AFTER RFA FOR HCC

Since the last edition of this book (Table 2), a number of studies (Table 3) have provided long-term data on local recurrences, distal intrahepatic recurrences, overall survival, and disease-free survival suggesting RFA as a viable option for patients who are not eligible for transplantation or resection. Ng et al. reviewed recurrence patterns after RFA for HCC in 209 patients and their association with survival *(85)*. Radiofrequency ablation was successful in 92.7% of the patients. The ablation was performed percutaneously ($n = 101$, 48.3%), laparoscopically ($n = 17$, 8.1%), and operatively ($n = 91$, 43.5%).

Table 2
Morbidity, Mortality, and Recurrence from RFA (1998–2001)

Study	*Year*	*Reference*	*No. of patients (HCC)*	*No. of lesions*	*Morbidity and mortality*	*Median F/U (months)*	*Recurrence at RFA site*	*Distant recurrence*
Buscarini	2001	*(69)*	88 (100%)	101	2.3% morbidity, 0 mortality 2 subcapsular hematoma	34	12 (11.8%)	Liver 29 (33.0%)
Nicoli	2001	*(70)*	79 (100%)	86	2.5% morbidity, 2.5% mortality 1 ascites, 1 liver failure 2 deaths from sepsis	NA	NA	NA
Bowles	2001	*(71)*	76 (33%)	328	19.7% morbidity, 1.3% mortality 3 bile leaks, 2 bile duct stricture, 1 bleeding, 2 grounding pad burns, 2 wound infections, 5 myoglobinuria, 1 death liver failure	15	30 (9%)	NA
Chung	2001	*(72)*	27 (15%)	85	3.7% morbidity, 0 mortality	14	4 (4.7%)	16 (59%)

(*Continued*)

Table 2
(Continued)

Study	*Year*	*Reference*	*No. of patients (HCC)*	*No. of lesions*	*Morbidity and mortality*	*Median F/U (months)*	*Recurrence at RFA site*	*Distant recurrence*
					1 delayed bleeding			
Bilchik	2000	*(73)*	68 (13.2%)	181	8.8% morbidity, 1.5% mortality 1 bile duct stricture, 3 abscesses, 1 bleeding, 1 diaphragmatic necrosis	12	5 (2.8%)	28 (41%)
Curley	2000	*(74)*	110 (100%)	149	8% morbidity, 0 mortality 1 delayed bleeding, 4 ascites, 2 pleural effusions, 1 persistent fever, 1 V-fib	19	4 (3.6%)	Liver 37 (33.6%) Extrahepatic 13 (11.8%)
Goldberg	2000	*(75)*	22 (18%)	23	4.5% morbidity, 0 mortality 1 grounding pad burn	NA	NA	NA
Siperstein	2000	*(76)*	43 (6.1%)	181	NA	12	22 (12.2%)	NA

Table 2
(Continued)

Study	*Year*	*Reference*	*No. of patients (HCC)*	*No. of lesions*	*Morbidity and mortality*	*Median F/U (months)*	*Recurrence at RFA site*	*Distant recurrence*
Wood	2000	*(77)*	84 (13%)	231	9.5% morbidity, 3.6% mortality 1 grounding pad burn, 1 delayed bleeding, 3 abscesses, 1 liver insufficiency, 1 heart attack, 3 deaths	9	15 (6.5%)	33 (39.3%)
Elias	2000	*(78)*	21 (0)	33	23.8% morbidity, 0 mortality 1 bile leak, 4 liver insufficiencies	17.3	1 (3.1%)	Liver 7 (21%) Extrahepatic 8 (24%)
Goletti	2000	*(79)*	7 (100%)	10	NA	6	0	Liver 2 (28.6%)
Cuschieri	1999	*(80)*	10 (20%)	32	0 morbidity, 10% mortality 1 death liver failure, hepatorenal	13	0	Liver 1 (10%)
Pearson	1999	*(81)*	92 (37%)	138	3.3% morbidity, 0 mortality	15	3 (2.2%)	NA

(*Continued*)

Table 2
(Continued)

Study	*Year*	*Reference*	*No. of patients (HCC)*	*No. of lesions*	*Morbidity and mortality*	*Median F/U (months)*	*Recurrence at RFA site*	*Distant recurrence*
					1 delayed bleeding, 2 abscesses			
Curley	1999	*(82)*	123 (39%)	169	2.4% morbidity, 0 mortality	15	3 (1.8%)	Liver 27 (21.9%)
					2 abscesses, 1 delayed bleeding			Extrahepatic 7 (5.7%)
Rossi	1998	*(83)*	37 (62%)	40	2.7% morbidity, 0 mortality	11	HCC	Liver 5(21.7%)
					1 capsular necrosis		1 (4.2%)	Extrahepatic 0
							Other	Liver 5 (35.7%)
							1(6.3%)	Extrahepatic 7 (50%)

Table 3
Morbidity, Mortality, and Recurrence from RFA (2003–2008)

Author	*Year*	*No. of patients*	*No. of lesions*	*F/U (months)*	*Major morbidity (%)*	*Mortality (%)*	*Effective necrosis (%)*	*Local recurrence (%)*	*DIH recurrence*	*1-Year OS*	*5-Year OS*	*1-Year DFS*	*5-Year DFS*
Kim et al. *(84)*	2008	133	200	NA	6.8	1.5	94.7	6.7	NA	92.3	46.5	21.3	16
Ng et al. *(85)*	2008	209	286	26	15.7	0.9	92.7	14.5	51.6	87.2	42	30	28.4
Choi et al. *(86)*	2007	570	674	30.7	1.9	0	96.7	10.9	51.9	95.2	58	26.5	21
Guglielmi et al. *(87)*	2007	98	145	24.9	8.1	0	85.5	21.3	36.7	76.7	NA	NA	NA
Takahashi et al. *(88)*	2007	171	NA	36.7	NA	0	NA	17	55.6	98.8	76.8	NA	NA
Zytoon et al. *(89)*	2007	40	48	24.1	NA	NA	NA	23	52.5	NA	NA	20	NA
Cabassa et al. *(90)*	2006	59	68	23.6	1.7	0	20–89[a]	NA	57.6	94.4	43	17.5	NA
Shibata et al. *(91)*	2006	74	83	27	0–2.8	0	93–95	20–22	NA	100–94	NA	34–22	NA
Lencioni et al. *(92)*	2005	187	240	24	2	0	90	4.9	49.7	97	48	51	19

(*Continued*)

Table 3
(Continued)

Author	*Year*	*No. of patients*	*No. of lesions*	*F/U (months)*	*Major morbidity (%)*	*Mortality (%)*	*Effective necrosis (%)*	*Local recurrence (%)*	*DIH recurrence*	*1-Year OS*	*5-Year OS*	*1-Year DFS*	*5-Year DFS*
Machi et al. *(93)*	2005	65	191	>16	NA	NA	NA	16.9	57.1	NA	39.9	NA	27.9
Raut et al. *(94)*	2005	194	289	34.8	12	0	97–100	4.6	NA	84.5	55.4	43.1	33.1
Tateishi et al. *(95)*	2005	664	2140	26	4	0	NA	NA	34–64[e]	92–95[f]	38–54[g]	NA	NA
Ruzzenente et al. *(96)*	2004	87	104	19.2	6.1	0	57–100[b]	19.3	34.4	NA	NA	NA	NA
Giorgio et al. *(97)*	2003	62	95	10	0	0	12–95[c]	10	6	NA	NA	NA	NA
Guiglielmi et al. *(98)*	2003	53	65	18	NA	0	36–91[d]	6.8	28.3	87	NA	NA	NA

Rec = Recurrence; DIH = distal intrahepatic; OS = overall survival; DFS = disease-free survival

[a]Influenced by size: <3 cm 88.6%; 3–5 cm 52.6%, >5 cm 20% ($p < 0.001$)

[b]Influenced by size: <3 cm 100%; 3–5 cm 87.7%; >5 cm 57.1% ($p = 0.02$)

[c]Influenced by size: <3 cm 95%; 3–5 cm 71%; >5 cm 12%

[d]Influenced by size: <3 cm 90.9%; 3–5 cm 74.4%; >5 cm 36.4% ($p = 0.01$)

[e]45% after percutaneous RFA; 34% after open RFA without resection; 64% after open RFA with resection

[f]91.8% in non-naïve patients and 94.7% in naïve patients

[g]38.2% in non-naïve patients and 54.3% in naïve patients

Overall mortality and morbidity were 0.9 and 15.7%, respectively. Overall survival at 1 year, 3 years, and 5 years were 87.2, 66.6, and 42%, respectively. At a medium follow-up of 26 months, there was a 14.5% local recurrence rate and a 10.4% distant extrahepatic metastatic recurrence. Same segment and different segment intrahepatic recurrences were 15.6 and 40.6%, respectively. Different segment intrahepatic recurrences tended to develop in patients with chronic Hepatitis C infection and have more advanced tumor as measured by larger size, multiple tumor nodules, higher α-feta protein level, and more advanced CLIP score (which includes Child-Pugh stage, tumor morphology and extension, serum AFB level, and portal vein thrombosis). Local recurrence was associated with worse survival. Ng et al. *(85)* showed that overall survival was best seen in those patients without recurrence compared to those with local recurrence at the RFA site (5 years, 83% vs 63.6%, $p = 0.031$). Those with same segment intrahepatic recurrences had significantly better overall survival compared to those with different segment intrahepatic recurrences (5 years, 42% vs 23%, $p = 0.022$). Those with extrahepatic disease had the worst outcome with 1-year survival of only 18.3% with all patients deceased by 14 months after RFA.

Takahashi et al. also showed that local recurrence decreased overall survival *(88)*. They retrospectively reviewed a cohort of 171 Child-Pugh class A cirrhotic patients who underwent RFA for early-stage HCC within Milan criteria. After a median follow-up of 36.7 months, 1-year, 3-year, and 5-year cumulative survival rates were 98.8, 91.1 and 76.8%, respectively. Local recurrence at the RFA site was 17%, while distant intrahepatic recurrence was 55.6%. Survival after local recurrence at 1 year, 3 years, and 5 years was 96.6, 74.8, and 42.1%, respectively, while survival without local recurrence during the same time period was 96.6, 94.6, and 84.4%, respectively ($p = 0.0002$). Univariate and multivariate analyses showed that low serum albumin (<3.5 g/dl), high range of PIVKA-II (prothrombin induced by vitamin K absence or agonist IIm >100 mAU/ml), multiple nodules, and tumor recurrence after initial curative RFA therapy were all risk factors for death.

Other authors have confirmed that incomplete ablation negatively affects survival. Guglielmi et al. showed that patients with complete and incomplete tumor response had a median survival of 27 and 8 months, respectively ($p < 0.01$) *(87)*. Complete response was dependent on tumor size ($\leqq$3 cm vs >3 cm, 98.1% vs 78.7%, $p = 0.005$), distance from major vessels (90.2% vs 73.3%, $p = 0.02$), and α-fetoprotein level ($\leqq$ or > 100 ng/ml, 89.2% vs 58.8%, $p = 0.002$). Child-Pugh class, α-fetoprotein level, and complete tumor response after RFA were risk factors for survival. Patients with Child-Pugh class A cirrhosis and α-fetoprotein level <100 ng/ml had a median survival of 38 months, those with Child-Pugh class B cirrhosis and α-fetoprotein level <100 ng/ml had a median survival of 22 months, and those with Child-Pugh class A cirrhosis and α-fetoprotein level >100 ng/ml had

a median survival of 9 months ($p < 0.01$). A high α-fetoprotein level was associated with the highest hazard ratio for death at 4.0, while incomplete tumor response had a hazards ratio of 3.8. Child-Pugh class B patients had a relative risk of death of 2.7 compared to Child-Pugh class A patients.

Patients who had previous treatment for HCC (non-naïve patients) have worse outcome after RFA treatment compared to patients who were undergoing RFA treatment for HCC for the first time (naïve patients). Tateishi et al. reviewed their experience from 1000 RFAs in 664 patients *(95)*. They compared overall survival in those patients receiving RFA as their initial treatment (naïve patients) to those who received RFA for recurrence after other treatments including hepatic resection, percutaneous ethanol injection, percutaneous microwave coagulation therapy, or transarterial embolization (non-naïve patients). The 1-year, 3-year, and 5-year survivals for naïve patients were 94.7, 77.7, and 54.3%, respectively. The 1-year, 3-year, and 5-year survivals for non-naïve patients were 91.8, 62.4, and 38.2%, respectively. In the naïve patients, tumor recurrence occurred in 53.9% of patients; however, the study did not delineate if these recurrences were local, same segment, or different segment intrahepatic recurrences. No distant recurrences were noted, except in two (1.5%) patients with neoplastic seeding. Significant differences in survival were observed in patients that were subdivided by Child-Pugh class ($p = 0.0004$), tumor size ($p = 0.0002$), and AFP level ($p = 0.01$).

In addition to local recurrence, Lencioni et al. found that survival of patients treated with RFA was dependent on Child class ($p = 0.006$) and tumor multiplicity ($p = 0.013$) *(92)*. Patients with Child class A cirrhosis and a single HCC lesion treated with RFA had a median survival of 65 months (5 years, 5 months) and a 5-year survival rate of 61%.

The rate of effective, complete tumor ablation ranges from 12 to 100% (Table 3). Tumor size appears to be the most critical factor influencing the chance for complete tumor ablation, which directly affects local recurrence rates. Complete ablation was possible in 88.6–100% of patients with HCC that were $\leq$3 cm, 52.6–87.7% of patients with tumors that were 3–5 cm in size, and only 12–57.1% of patients with tumors > 5 cm in size ($p < 0.001$–0.02) (90, 96–99). Tumor size was associated with poor survival as median survival for patients with tumor diameters $\leq$3 cm, 3–5 cm, and $\geq$ 5 cm were 25.5, 23.3, and 11.4 months, respectively ($p = 0.05$) *(90)*. This suggests that other treatment modalities, such as transcatheter chemoembolization (TACE) or the percutaneous ethanol injection may be necessary as an adjunct to RFA in patients with HCC lesions >5 cm.

As noted previously, intrahepatic recurrence after RFA appears to occur more often at distant intrahepatic locations (6–57.6%), as opposed to local recurrence at the RFA site (4.6–23%) (Table 3). This may be due to the multifocal nature of the HCC or may suggest that synchronous multifocal

disease may be present at the time of RFA treatment that was not previously detected radiographically. Close follow-up after RFA treatment is necessary to detect new lesions that may be further treated. As mentioned previously, Ng et al. noted that intrahepatic recurrences at different segments usually develop in patients with chronic Hepatitis C infection, more advanced tumor as measured by larger size, multiple tumor nodules, higher α-feta protein level, and more advanced CLIP score *(85)*. Adjuvant therapy may be considered in this subset of patients.

12. COMPLICATIONS FROM RFA

Since 2002, four publications have evaluated the complications associated with RFA for liver tumors *(100–103)*. From a total of 8,916 patients, these studies revealed an overall complication rate of 2.2–10.6% and an overall mortality rate of 0.3–1.4% after RFA for liver tumors. Table 4 outlines the complications associated with RFA. The complications were categorized as tumor-related, liver-related, surgical, general, or minor complications.

Mulier et al. reviewed 82 independent reports of RFA of liver tumors in 3,670 patients *(100)*. They found an overall complication rate of 8.9% and mortality rate of 0.5%. The complications after a percutaneous, laparoscopic, simple open, and combined open approach were 7.2, 9.5, 9.9, and 31.8%, respectively, and the mortality rates were 0.5, 0, 0, and 4.5%, respectively.

De Baere et al. reviewed their experience with 312 patients having 582 liver tumors, 19.8% of which were HCC and 80.2% of which were metastatic tumors. Major complications occurred in 5.7% of patients and death occurred in 1.6% of patients *(101)*.

The incidence of complications from RFA may be dependent on who is performing the procedure. Livraghi et al. also conducted a questionnaire survey from 41 Italian centers that performed 3,554 RFA in 2,320 patients, 69.4% of whom were diagnosed with HCC and 29.9% of whom were diagnosed with metastatic cancer to the liver *(102)*. Major complications occurred in 2.2% of patients and death occurred in 0.3% of patients. When they compared different types of centers, they found that the rate of combined death and major complications was 16.7 per 1,000 patients at radiologic centers, 23.0 per 1,000 patients at medical centers, and 60.2 per 1,000 at surgical centers ($p = 0.01$), suggesting that more experience with percutaneous image-guided techniques helps decrease complications.

Kasugai et al. conducted a questionnaire survey from 43 departments of 38 facilities that performed a total of 3,891 RFA in 2,614 patients *(103)*. The procedure was performed percutaneously in 97.2% of patients, laparoscopically in 0.9% of patients, and operatively in 1.9% of patients. Complications

Table 4
Complications*

Tumor-related complications
–Rapid tumor progression (0.11–4.6%) (96)
–Needle track seeding (0–12.5%) (36)
–Peritoneal seeding (0.11%)
–Rumor rupture

Liver-related complications
–Bile leak/biloma (0.06–0.96%)
–Liver abscess (0.2–2.0%)
–Portal vein thrombosis (0.2–0.8%)
–Hepatic vein thrombosis (0.1–1.4%)
–Liver dysfunction or failure (0.08–0.78%)
–Bile duct stricture/stenosis (0.06–0.5%)
–Liver infarction (0.038–0.06%)
–Subcapsular hematoma (0.15–0.5%)

Surgical complications
–Hemorrhage (0.3–1.6%)
–Bowel perforation (colon > small bowel, stomach) (0.06–0.3%)
–Cholecystitis (0.06–0.1%)
–Peritoneal abscess (0.1%)
–Skin/abdominal wall burns (0.1–0.38%)
–Burn at grounding pad site (0.6%)

General complications
–Pleural effusions (0.2–2.3%)
–Ascites (1.3%)
–Pneumothorax (0.15–0.8%)
–Pulmonary embolism (0.06%)
–Pneumonia (0.1%)
–Sepsis (0.06–0.1%)
–Myocardial infarction (0.038–0.1%)
–Transient renal failure (0.1–0.3%)

Minor complications
–Fever or febrile syndrome
–Moderate pain

*Percentages obtained from Mulier et al. *(100)*, de Baere et al. *(101)*, Livraghi et al. *(102)*, Kasugai et al. *(103)*, Ruzzenente et al. *(96)*, and Llovet et al. *(36)*

were observed in 7.9% of patients and 0.3% of patients died within 3 months of the RFA.

Tumor seeding along the needle track is a major concern after RFA. Stigliano et al. reviewed the literature on the risk of tumor seeding following percutaneous diagnostic approaches for HCC *(104)*. They found that the median risk of seeding was 2.29% (0–11%) for patients undergoing liver biopsy only, 0.61% (0–5.56%) for patients being treated with RFA without biopsy, and 0.95% (0–12.5%) for patients undergoing RFA with biopsy. Given the risk of tumor seeding, the authors concluded that biopsy of suspected HCC in cirrhotic patients should not be performed. The highest risk of seeding was reported by Llovet et al. in 4 of 32 patients (12.5%) undergoing percutaneous RFA for HCC by the Barcelona Clinic Liver Cancer Group *(36)*. This study was limited by the number of patients ($n = 32$); nevertheless, they identified subcapsular tumor location ($p = 0.009$), poorly differentiated tumor ($p = 0.02$), and elevated baseline AFP levels ($p = 0.02$) as significant independent variables predicting tumor seeding. They utilized the cooled-tip single electrode system from Radionics. Seeding occurred outside the hepatic capsule in three patients and in the paracolic gutter in one patient at 4–18 months after RFA. It is unclear if the increased risk of seeding in the current study was related to the antecedent liver biopsy in 84% of cases or whether the saline cooled-tip design predisposes to such an event compared to the retractable arrays with the RITA or RadioTherapeutics devices.

Tumor recurrence may occur very rapidly after RFA treatment. Rapid tumor progression, as defined by wide neoplastic spread to adjacent liver segments within 1–2 months, was noted in 3.2–4.5% of patients after RFA *(96*, *105)*. Ruzzenente et al. identified patients with elevated preoperative AFP levels (>200 kU/L), poorly differentiated tumors, and tumors located near the main portal branch as risk factors for rapid tumor progression after RFA *(96)*. Zavaglia et al. suggested that dissemination of tumor emboli through hepatic vessels may occur as a consequence of gas formation induced by the heat *(105)*.

Complications may occur due to the proximity of HCC lesions close to surrounding structures. Approximately 9% of HCCs may not be ablated percutaneously due to unfavorable location of the tumor, such as adjacent, viscera, portal structure, or unfavorable path due to ribs, lung, or diaphragm *(62*, *92)*. Lesions on the dome of the liver can be attempted percutaneously with the assistance of artificial ascites *(106)*. Otherwise, the open RFA approach has some advantages. Intraoperative ultrasound allows for direct placement of ultrasound probe onto the liver surface which improves visualization of the tumor. Also, with the open approach, other procedures can be performed, including liver resection and Pringle maneuver, which, theoretically, augments the RF ablation zone by eliminating the heat–sink phenomenon caused by the hepatic arterial and portal venous flow *(107*, *108)*.

13. SUMMARY

RFA is currently a treatment option for patients with HCC or metastatic liver tumors. Indications for RFA for HCC include patients with HCC, but who are not transplant candidates or have unresectable disease due to multilobar disease. Some centers also have reported use of percutaneous or laparoscopic RFA as a bridge to liver transplantation, although this remains controversial. Indications and contraindications based on size, number, and location are quite variable in the literature. In addition, patient preference must be considered, especially in patients who have well-compensated liver disease (Child class A) and small lesions (<3 cm) or those who prefer a minimally invasive approach to treat their HCC. Percutaneous RFA is fast, effective, less invasive, requires a shorter hospital stay, and is associated with low mortality. If found early in patients with less advanced cirrhosis (Child-Pugh class A) and small tumors ($\leqq$3 cm), treatment of HCC by RFA without local recurrence is associated with long-term survival that is as comparable to surgical resection and liver transplantation. Unfortunately, the median size of HCC treated in the United States is 8 cm, compared with a median size of 3.5 cm found in Japan ($p < 0.001$) where screening programs help identify early HCC in the endemic hepatitis C population *(109)*. Major complications occur in less than 10% of cases in most series, with minimal to no mortality. Compared to PEI, treatment of HCC with RFA is associated with improved survival and less recurrence. The addition of TACE to RFA may improve survival even further.

Liver transplantation and hepatic resection remain the best treatment options for HCC for those who meet transplant criteria or are good operative candidates. Five-year survival after transplantation ranges from 61 to 75% *(3)*, while 5-year survival after surgical resection ranges from 31 to 93% *(3*, *4*, *109*, *110)*. For those patients who are not transplant or operative candidates, RFA remains a viable option. Five-year survival rates after RFA range from 38.2 to 84.4% (Table 3), with the best results from a subset of patients with small, solitary HCC in Child class A cirrhotic who do not develop local recurrence after RFA treatment (5-year survival of 84.4%) *(88)*.

Although randomized trials are lacking, application of RFA to treat patients with early HCC while awaiting liver transplant appears promising. In general, RFA is best applied to tumors <3 cm in size. Local recurrences at the RFA site have been documented to occur in 4.6–26.5% of cases and can usually be diagnosed by follow-up imaging studies. However, recurrences elsewhere in the liver (6–57.6%) or at extrahepatic sites (0–10.4%) will occur depending on the length of the follow-up and suggest that trials with a multimodality approach may be warranted.

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17 Resection of Hepatocellular Carcinoma

Ronnie Tung Ping Poon MS, PhD, FRCS (Edin), FACS

CONTENTS

ABSTRACT

Liver resection remains a first-line curative treatment for HCC in patients with non-cirrhotic liver or cirrhotic liver with preserved liver function. New strategies, such as portal vein embolization and combined resection/ablation, have extended the indication of hepatic resection for HCC. Laparoscopic hepatectomy may further enhance the benefit of resection for HCC by reducing blood loss and morbidity. However, a high postoperative recurrence rate remains a major problem limiting the long-term survival. New trials

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involving adjuvant therapies are in progress, with the aim of reducing these recurrences.

Key Words: resection; RFA; laparoscopy; adjuvant therapy

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies, ranking fifth in frequency among all malignancies in the world *(1)*. Despite recent advances in other curative treatment options such as liver transplantation and radiofrequency ablation, surgical resection remains the mainstay of curative treatment for HCC because of the limited availability of liver grafts and the limitation of tumor size amenable to ablation treatment *(2, 3)*. The majority of patients with HCC have associated liver cirrhosis related to hepatitis B or C viral infection, which often contraindicates surgical resection because of impaired liver function. Furthermore, many patients with HCC present with advanced tumor and only about 20–30% of patients with HCC have resectable disease on presentation *(4)*. The wider utilization of screening program in high-risk patients such as those with cirrhosis and chronic hepatitis B virus carriers has resulted in early detection of small HCCs in recent years and may improve the chance of surgical treatment *(5)*.

2. INDICATIONS FOR RESECTION

An HCC with diameter of less than 5 cm is the best candidate for resection because of increased risk of additional nodules or vascular invasion and consequently incomplete resection with larger HCCs *(6, 7)*. However, it has been shown that patients with a large solitary HCC (Fig. 1) can undergo resection safely with appropriate selection in terms of liver function reserve,

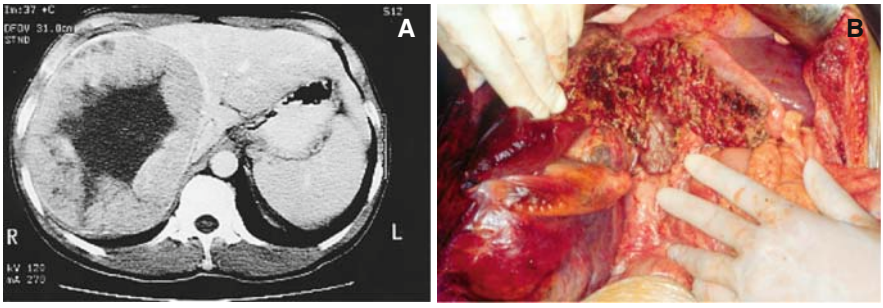

Fig. 1. A solitary large HCC in the right lobe of the liver stretching the middle hepatic vein (**A**) was resected by extended right hepatectomy (**B**).

and reasonable long-term survival results can be achieved that appear to be much better than any nonsurgical treatmentsthat can offer *(8–10)*. In the recent guideline of the American Association for the Study of Liver, liver resection is recommended for solitary tumor irrespective of tumor size, but multifocal HCCs or HCCs with vascular invasion are not considered as favorable surgical candidates *(11)*. However, studies from experienced liver surgical groups have shown that while the presence of multiple tumor nodules or vascular invasion in major intrahepatic venous branches is associated with worse prognosis compared with those patients with solitary tumor without vascular invasion, surgical resection is still associated with the best long-term survival compared with nonsurgical treatments *(10, 12)*. Bilobar HCC used to be a contraindication for resection, but a recent study suggested that patients with a predominant mass in one lobe and one or two small tumor nodules in the other lobe may benefit from combined resection of the predominant tumor and ablation or chemoembolization for the contralateral nodules *(13)*. Hence, with increasing safety of resection and larger experience in specialized centers, liver surgeons are generally more aggressive in offering resection for HCC patients provided that the tumors are anatomically resectable and the liver function reserve is adequate. However, the presence of distant metastasis, main portal vein thrombosis, or inferior vena cava thrombosis is considered by most as definite contraindication for resection. As most of the symptoms of HCC can be palliated with medical treatments and liver resection remains a major surgical procedure with significant risk, unlike the cases of other gastrointestinal cancers such as colorectal or gastric cancers, there is no role of palliative resection for HCC.

3. PREOPERATIVE ASSESSMENT

The routine preoperative imaging prior to liver resection includes a chest X-ray or contrast computed tomography (CT) scan of abdomen to exclude lung metastasis and a helical contrast CT scan of abdomen (or magnetic resonance imaging) to assess the tumor status. The CT scan of abdomen provides important information not only on the tumor size, number, and any vascular invasion but also on the relationship of the tumor to intrahepatic portal pedicles and hepatic veins. Nonhistological criteria of diagnosis of HCC based on the typical arterial enhancement and portal venous contrast washout are well accepted for diagnosis of HCC in cirrhotic liver *(11)*. Preoperative biopsy is generally not necessary and may risk needle track seeding, which is rare (1% risk) but may convert a curable case to an incurable case.

One of the main risks of liver resection in cirrhotic liver is liver failure, which is particularly worrisome in patients requiring major resection for large or multifocal tumors. Proper assessment and selection in terms

of liver function reserve is the key to safe resection of HCC. Liver function reserve is most commonly assessed by a combination of Child's classification and liver biochemistry. Only Child class A patients are suitable for a major hepatectomy removing three or more segments of the liver. Relatively compensated Child B patients may be suitable for minor hepatectomy (wedge resection or segmentectomy), whereas Child C cirrhotic patients are contraindicated for resection. The bilirubin and albumin levels reflect the excretory function and the synthetic function of the liver, respectively. Platelet count is also important as it reflects the severity of portal hypertension. Patients with clinical evidence of portal hypertension such as history of variceal bleeding or ascites generally do not tolerate hepatic resection except peripheral wedge resection. While the Child's classification provides a general guideline to the indication for major or minor hepatic resection, the perioperative results of patients within Child class A can vary significantly, suggesting that further refined test may help to improve patient selection. In some centers, special tests of excretory function of the liver, such as indocyanine green clearance test and galactose elimination capacity, are used to further refine the assessment of liver function *(14, 15)*. However, these specific liver function tests reflect the function of the whole liver, while the risk of postoperative liver failure depends on the liver function reserve of the liver remnant. The indocyanine green clearance test is the more commonly used test of liver function in Eastern centers, and depending on individual center's practice, an ICG retention rate at 15 min (ICG R15) of 10–20% is considered the upper limit for safe major hepatic resection *(16, 17)*. Recently, the Model for End-Stage Liver Disease (MELD) score, which was adopted by the United Network for Organ Sharing in the United States to prioritize organ (liver) allocation, has been shown to predict perioperative outcome in patients undergoing liver resection. Cirrhotic patients with greater MELD scores ($\geq$9) are at increased risk for postoperative morbidity and mortality *(18, 19)*.

Extended right or left hepatic resection can be performed even in the presence of cirrhosis, provided patients are carefully selected in terms of liver functional reserve *(20)*. Recent advance in CT volumetry allows assessment of the volume of the future liver remnant to provide an anatomical guideline to the safety of major hepatic resection in addition to the functional reserve *(21)*. In patients with inadequate liver remnant volume for a right or extended right hepatectomy, preoperative portal vein embolization can be employed to induce hypertrophy of the liver remnant before resection. One prospective nonrandomized study suggested that preoperative right portal vein embolization could induce significant hypertrophy in patients with liver fibrosis or mild cirrhosis, reducing the incidence of complications compared with right hepatectomy without preoperative portal vein embolization *(22)*. However, it is still controversial regarding which index of the future liver remnant volume should be used. Some surgeons use the actual total liver volume,

defined as the volume of the patient's liver measured directly on CT images minus tumor volume *(21, 22)*, while others use the estimated ideal liver volume, which is calculated by a formula based on a linear correlation between total liver volume and body weight or body surface area in healthy subjects *(23, 24)*. There is also no consensus on the adequate future liver remnant volume in cirrhotic patients. For patients with cirrhosis, some authors suggested that portal vein embolization is indicated when the future liver volume is ≤40% of total liver volume *(22, 25)*. It usually takes 4–6 weeks before liver hypertrophy is adequate for resection. Some surgeons perform transarterial chemoembolization 1–2 weeks before portal vein embolization, aiming to prevent tumor progression during the period between portal vein embolization and planned hepatectomy, and enhance the effect of portal vein embolization by embolizing possible arterioportal shunts *(26)*. Depending on the severity of underlying cirrhosis, some patients may not undergo adequate liver hypertrophy after portal vein embolization, which should be considered a contraindication for major hepatic resection.

4. TECHNIQUES OF LIVER RESECTION

Liver resection is now a safe operation even in cirrhotic liver provided that patients are selected carefully and the operations are performed in specialized liver surgery units. There are several advances in surgical techniques that have improved surgical outcome, in particular techniques that help to reduce bleeding during liver transection. Reduced blood loss and perioperative blood transfusion are the main factors for decreased operative morbidity and mortality in recent years *(27)*.

One of the most important advances is the thorough understanding of the segmental anatomy of the liver, which can be delineated using intraoperative ultrasound during operation. The delineation of a proper transection plane is important not only for adequate tumor-free margin in resection of liver tumors but also to avoid inadvertent injuries to major intrahepatic vessels or bile duct pedicles. In general, a tumor-free margin of 1 cm is considered necessary for curative purpose, although a recent randomized study suggested that a wider resection of 2 cm may improve the long-term outcome of patients *(28)*. However, in cirrhotic patients with borderline liver function reserve, preservation of liver parenchyma may take priority over a wide resection margin *(29)*. Because of the pattern of intrahepatic spread of liver cancer cells along segmental portal vein pedicle, segmental resection may improve the chance of tumor clearance compared with a nonanatomical wedge resection *(30)*. Intraoperative ultrasound allows localization of the segmental portal pedicle, and some surgeons use dye injection into the segmental portal vein to stain the segment and more clearly delineate the transection plane before resection *(31)*.

Hepatic transection is difficult in cirrhotic liver due to the fibrotic nature of the liver tissue and the presence of bleeding tendency. The conventional technique of liver transection is the finger fracture or Kelly clamp crushing technique, which involves crushing of liver parenchyma by fingers or Kelly clamp to isolate vessels and bile ducts for ligation *(32)*. Clamp crushing technique is still one of the most widely used techniques of liver transection nowadays. However, in many centers, including the author's center, ultrasonic dissection using the Cavitron Ultrasonic Surgical Aspirator (CUSA, Tyco Healthcare, Mansfield, MA) has become the standard technique of liver transection. With this technology, the liver parenchymal tissue is fragmented with ultrasonic energy and aspirated, thus exposing vascular and ductal structures that can be ligated or clipped with *(33)*. Other transection techniques include water jet, harmonic scalpel, TissueLink, Ligaure, and radiofrequency-assisted techniques, employing various energies to fragment liver parenchyma and to coagulate transection surface for hemostasis *(34)*. The result with each transection technique is significantly affected by the individual surgeon's experience with the respective technique.

Inflow occlusion by clamping of the portal triad (Pringle maneuver) is frequently used to reduce bleeding during hepatic transection. However, there is a limit to the duration that the Pringle maneuver can be applied. Prolonged application of the Pringle maneuver for a total of more than 120 min may have deleterious effects on the liver function *(35)*. Other surgeons have used total hepatic vascular exclusion instead of the Pringle maneuver to reduce blood loss in major liver resection. However, hepatic vascular exclusion is associated with unpredictable hemodynamic intolerance and increased postoperative complications compared with the Pringle maneuver *(36)*. A major source of bleeding during liver transection is hepatic vein branches in the deeper part of the transection plane. Such bleeding can be reduced by low central venous pressure achieved by a combination of posture change, fluid restriction, diuretics, vasodilators, and anesthetic agents that produce vasodilatation *(37)*. The central venous pressure should be lowered to less than 5 mmHg, provided that the hemodynamic status is stable. One concern of low central venous pressure is the increased possibility of air embolism. However, clinically significant air embolism is seldom observed, and the benefit of reduced bleeding with low central venous pressure outweighs the risk of air embolism.

In recent years, liver resection by laparoscopic approach becomes feasible in experienced centers due to improvement in instruments for laparoscopic liver transection (Fig. 2). Small HCCs in anterior segments and left lateral segments are most amenable for laparoscopic resection, which has the advantage of minimal invasiveness with less pain, shorter hospital stay, and possibly reduced blood loss in case–control studies compared with open resection *(38)*. Resection of lesions in posterior segments and major resection are technically more demanding but feasible in very experienced

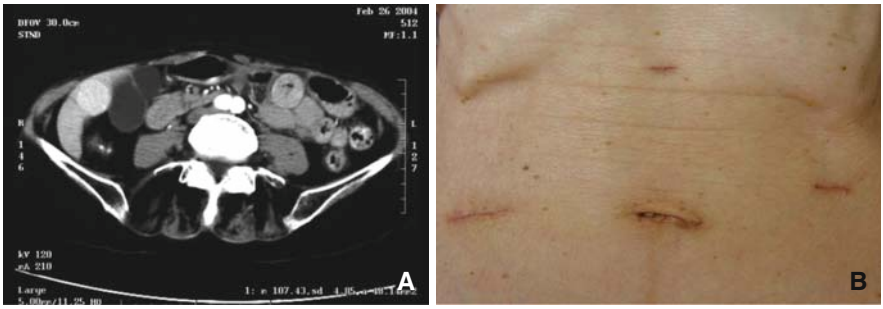

Fig. 2. A small peripheral HCC in segment 6 (**A**) was resected by totally laparoscopic approach. Patient was discharged the next day with only small 1-cm wounds (**B**).

hands. Preliminary data from small series suggest that laparoscopic resection of small HCC is associated with similar oncological clearance and mid-term survival results compared with open resection *(39)*. Further studies, preferably prospective randomized trials, are needed to confirm the oncological efficacy and benefit of laparoscopic resection compared with open resection.

For patients with bilobar HCC, combined resection and radiofrequency ablation is a new strategy to increase the chance of curative treatment. If the patient has a large tumor in one lobe and smaller lesion(s) in the other lobe, resection of the large tumor and ablation of smaller tumor(s) can be performed. For patients with multifocal tumors associated with cirrhosis and borderline liver function, resection of peripheral lesions and ablation of central lesions may allow parenchymal preservation. A recent study suggested that combined resection and ablation did not increase the operative morbidity compared with resection alone, and the long-term survival results appeared comparable to that of resection alone *(40)*.

5. CURRENT RESULTS OF HEPATIC RESECTION

With advances in surgical techniques and perioperative management, near-zero hospital mortality rate after resection of HCC has been reported from very experienced centers *(41, 42)*. In most major centers, an operative mortality rate below 5% is the current standard even for major hepatic resection in Child A cirrhosis. However, the morbidity rate remains high, about 30–40% even in experienced centers *(27, 42)*. Serious complications such as liver failure, intra-abdominal hemorrhage, bile leakage, and intra-abdominal sepsis are less frequent nowadays, but wound infection and pulmonary complications remain common *(27)*.

The long-term survival results after resection of HCC have also improved in recent years *(43)*. The overall 5-year survival after resection of HCC, inclusive of small and large HCCs, in large series in the literature is in

Table 1
Long-Term Survival After Resection of HCC

Author (year)	*No. of patients*	*5-Year disease-free survival (%)*	*5-Year overall survival (%)*
Takenaka (1996) *(44)*	280	29	50
Makuuchi (1998) *(45)*	306	13	47
Lise (1998) *(46)*	100	26	38
Fan (1999) *(47)*	211	27	37
Grazi (2001) *(48)*	264	28	41
Capussotti (2005) *(49)*	216	25	34

the range of 35–50% (Table 1) *(44–50)*. A 5-year survival of 50% can be expected in more recently operated patients *(42)*. Reduction in blood loss and hence perioperative blood transfusion is a significant factor in the improved long-term survival *(43)*. Perioperative transfusion has been found to have adverse impact on the long-term survival after resection of HCC, probably by an inhibitory effect on immune system that leads to increased risk of recurrence *(47)*. The long-term disease-free survival remains poor, less than 30% at 5 years in most series (Table 1). The incidence of postoperative recurrence at 5 years is in excess of 70% in most series, due to metastatic lesions or multicentric recurrences in the liver remnant *(50)*. Adverse tumor factors such as the presence of vascular invasion or microsatellite nodules are dominant risk factors of recurrence in many studies, suggesting that microscopic metastasis is an important cause of recurrence. The presence of cirrhosis also adversely affects the long-term prognosis because it predisposes to multicentric recurrence. The activity of hepatitis virus at the time of resection has also been linked to increased risk of recurrence *(51)*. Table 2 summarizes the risk factors for tumor recurrence.

6. ADJUVANT THERAPIES

Neoadjuvant transarterial chemoembolization or postoperative adjuvant systemic/regional chemotherapy has so far failed to prevent recurrence in prospective clinical trials *(50)*. Recent studies have demonstrated the efficacy of some new modalities of adjuvant therapy in the prevention of postoperative recurrence after resection of HCC, such as polyprenoic acid *(52)*, intra-arterial radioactive iodine *(53)*, and adoptive immunotherapy *(54)*. However, none of these have been further validated by randomized trials with large sample size. Recently a few randomized controlled trials from Asian centers suggested benefits of interferon as adjuvant therapy after resection of HCC in reducing recurrence and prolonging survival *(55–57)*.

Table 2
Risk Factors for Tumor Recurrence After Resection of HCC *(49)*

Tumor factors	*Host factors*	*Operation factors*
Tumor size >5 cm	Cirrhosis	Positive margin
Multiple tumors	Active hepatitis	Nonanatomical resection
Macroscopic portal vein branch invasion	High viral load	Tumor manipulation
Microscopic venous invasion		Perioperative blood transfusion
Microsatellite nodules		
High-grade tumors		
Tumor rupture		
Advanced pTNM stage		
High AFP level		

pTNM, pathological tumor-node-metastasis; AFP, α-fetoprotein

Interferon has both antiviral activity and anti-tumor activity via inhibition of angiogenesis. However, interferon is associated with significant toxicity and a substantial portion of patients may not be able to tolerate the treatment. Furthermore, a Western randomized controlled trial failed to demonstrate the benefit of interferon in overall reduction of recurrence after resection of HCC, though it may reduce late recurrence in HCC patients purely related to hepatitis C virus receiving effective treatment *(58)*.

Thus far, there is no well-established adjuvant therapy after resection of HCC. The association of high viral load at the time of resection with increased postoperative recurrence after resection of hepatitis B virus-related HCC suggested that antiviral therapy may be useful in reducing multicentric recurrence, though no data are available in the literature yet. Another potential approach is to use molecular targeting drugs that may inhibit growth of micrometastases *(59)*. Sorafenib, an agent that targets both HCC cell proliferation and angiogenesis, has been proven to be effective in prolonging survival of patients with advanced HCC *(60)*. Currently a large-scale phase III randomized trial is ongoing to test the efficacy of sorafenib as adjuvant therapy after resection of HCC.

Aggressive treatment of recurrent tumors by re-resection or nonsurgical modalities such as transarterial chemoembolization and ablation therapy can result in prolonged survival even after the development of recurrent tumors *(61)*. This is the most practical way to increase patient survival prior to the availability of effective adjuvant therapy. For patients who have undergone resection of small HCC and develop intrahepatic recurrence, salvage liver transplantation is a potential option. With regular surveillance by CT scan, a high proportion of patients are eligible for transplantation when they develop

intrahepatic recurrence *(62)*. A French study has suggested that the perioperative and long-term survival outcomes of salvage transplantation were similar to those of primary liver transplantation *(63)*.

7. CONCLUSIONS

With careful patient selection, optimal surgical techniques, and meticulous operative care, the current operative mortality of hepatic resection in experienced centers is less than 5%. With the improved perioperative outcome and long-term survival, liver resection remains a first-line curative treatment for HCC in patients with noncirrhotic liver or cirrhosis with preserved liver function. New strategies such as portal vein embolization and combined resection/ablation have extended the indication of hepatic resection for HCC. Laparoscopic hepatectomy may further enhance the benefit of resection for HCC by reducing blood loss and morbidity. However, a high postoperative recurrence rate remains a major problem limiting the long-term survival. Further research should focus on the development of effective adjuvant therapy to prevent tumor recurrence.

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18 Liver Transplantation for Hepatocellular Carcinoma

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CONTENTS

ABSTRACT

Hepatocellular carcinoma is one of the most common cancers worldwide, and its incidence in the United States is increasing. Although complete surgical resection or ablation can provide cure for a small minority of patients with this disease, the vast majority develop HCC in the setting of cirrhosis. Thus, standard therapies aimed at localizable tumor(s) may fail to treat

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synchronous lesions present in other parts of the liver and do nothing to address the underlying liver disease itself. The underlying liver disease and late presentation of HCC have historically limited the options available for treatment in the majority of patients. Aggressive screening of patients at high risk for HCC has led to earlier diagnosis, making complete removal at such an early point feasible; however, it has become apparent that recurrence in these patients is virtually 100% if follow-up is long enough. Thus, the optimal treatment of HCC should include not only removal of all tumor(s) but also correction of the underlying hepatic disease process that incites their formation.

Key Words: Hepatocellular carcinoma; Liver transplantation; Molecular profiling; Microdissection-guided genotyping; Mutational analysis; Loss of heterozygosity; Locoregional therapy for hepatocellular carcinoma; Transarterial chemoembolization (TACE); Radiofrequency ablation (RFA); Yttrium-90, UNOS/Milan criteria

1. INTRODUCTION

Hepatocellular carcinoma is one of the most common cancers worldwide, with an estimated annual incidence of approximately one million cases. The incidence of HCC in the United States is increasing, related for the most part to hepatitis C *(1, 2)*. Although complete surgical resection or ablation can provide cure for a small minority of patients with this disease, the vast majority develop HCC in the setting of cirrhosis at a rate of 3–4% per year, the implications of which are twofold *(3–10)*. First, the underlying liver disease is frequently the limiting factor in making treatment decisions, as patients with advanced cirrhosis and/or portal hypertension often cannot tolerate therapies targeted against the tumor. Second, the underlying liver disease essentially constitutes a premalignant condition. Thus, standard therapies aimed at localizable tumor(s) may fail to treat synchronous lesions present in other parts of the liver; further, they do nothing to address the underlying liver disease itself. As a consequence, the diseased liver parenchyma can give rise to new lesions indefinitely.

The underlying liver disease and late presentation of HCC have historically limited the options available for treatment in the majority of patients; the median survival from time of diagnosis to death has been reported to be 6 months in untreated patients *(11)*. Aggressive screening of patients at high risk for HCC has led to earlier diagnosis, making complete extirpation of the tumor(s) at such an early point feasible; however, it has become apparent that recurrence in these patients is virtually 100% if follow-up is long enough *(12–14)*. Thus, the optimal treatment of HCC should include not

only removal of all tumor(s) but also correction of the underlying hepatic disease process that incites their formation. Currently the only treatment which can achieve both of these goals is complete hepatectomy and orthotopic liver transplantation (LT).

2. HISTORICAL ASPECTS OF LIVER TRANSPLANTATION FOR HCC

With the successful development of liver transplantation, there was hope that this procedure would provide a new and potentially curative treatment for patients with HCC since LT allows the removal of tumors deemed unresectable, while at the same time expunging the premalignant liver tissue *(15)*. However, as experience grew, the initial enthusiasm faded as early recurrences developed in the majority of patients *(16–23)*. In the early Pittsburgh experience, Iwatsuki et al. observed tumor recurrence in 72% (13/18) of patients transplanted for unresectable HCC but in none of the 13 patients found to have incidental tumors *(17)*. The Cincinatti Transplant Tumor Registry reported a 39% recurrence rate for non-incidental tumors, with only 9% (34/365) of patients surviving tumor-free for more than 2 years *(21)*. Similarly, Ringe reported a 25% tumor-free survival rate in 52 patients at a median follow-up of 19 months *(23)*. A statistically significant correlation between pTNM stage and actuarial survival was demonstrated in these early series, a trend which has been verified by a number of investigators *(23–27)*.

A review of data collected by the United Network for Organ Sharing (UNOS) for all cadaveric liver transplants performed in the United States confirmed the inferior outcomes for patients transplanted with HCC compared to those with other diagnoses. Such poor outcomes led to the exclusion of these patients at a number of transplant centers and was, until recently, considered a contraindication to LT by the Centers for Medicare & Medicaid Services. Without this approval, Medicare and consequently most third-party payors denied financial reimbursement for transplantation for those with HCC, effectively eliminating any chance for prolonged survival or cure in these patients. Despite this, a number of transplant centers continued to perform transplants in these patients, obtaining excellent results in some. It eventually became apparent that accurate diagnosis and staging could identify subgroups of patients for whom LT is curative or provides long-term, tumor-free survival; this led to a change of policy by the Centers for Medicare & Medicaid Services in 2001 to offer reimbursement for LT in patients with HCC under the following strict circumstances:

1. The patient is not a liver resection candidate
2. The patient's tumor(s) is less than or equal to 5 cm in diameter

3. There is no macrovascular involvement
4. There is no identifiable extrahepatic spread of tumor to surrounding lymph nodes, lungs, abdominal organs, or bone.

While these criteria were narrow, it was an appropriate beginning.

3. RISK FACTORS

Chronic active hepatitis B infection is one of the most common causes of HCC worldwide, particularly in the setting of cirrhosis. Likewise, hepatitis C increases the risk of HCC, and in the Western hemisphere is currently the most commonly associated condition *(28)*. Other types of post-necrotic cirrhosis also have a high association with HCC (e.g., hemochromatosis, tyrosinemia, and alpha-1 antitrypsin deficiency), but the overall incidence of these diseases is significantly less than viral hepatitis, making their total occurrence less. Cholestatic liver diseases such as primary biliary cirrhosis, primary sclerosing cholangitis, and biliary atresia rarely give rise to HCC; the association between alcohol-induced cirrhosis and HCC is in between these extremes.

A number of studies have demonstrated the benefit of screening for HCC in high-risk patient populations *(12–13)*. This typically consists of serial measurements of serum alpha-fetoprotein levels and imaging of the liver by ultrasound, computerized tomography (CT), or magnetic resonance imaging (MRI). However, this is not a widespread practice in the United States (as opposed to the developed Asian countries such as Japan) due to the lack of demonstrated cost effectiveness.

A presumptive diagnosis of HCC is often based on characteristic CT findings such as hypodensity on noncontrast and/or portal venous phases, with tumor enhancement in the arterial phase *(29)*. When there is doubt as to the diagnosis, the diagnosis may be confirmed by biopsy. If portal or hepatic vein thrombosis is present on preoperative imaging studies, percutaneous biopsy of the thrombus can be performed to differentiate bland from tumor thrombus. (This differentiation can often be made on imaging as bland thrombus does not enhance on arterial imaging but tumor thrombus often does.) Patients with malignant, venous thrombosis should not routinely be transplanted as the results are uniformly poor resulting in rapid recurrence and death from HCC, usually within the first postoperative year *(30)*.

4. STAGING OF HCC

Since the number of organs available for transplantation is inadequate to meet the demand, the selection criteria for potential transplant candidates

Table 1
pTNM Staging System for Hepatocellular Carcinoma

T	*Primary tumor*
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Solitary, ≤2 cm, no vascular invasion
T2	Solitary, ≤2 cm, vascular invasion; multiple, one lobe, ≤2 cm, no vascular invasion; or solitary, >2 cm, no vascular invasion
T3	Solitary, >2 cm, vascular invasion; multiple, one lobe, <2 cm, vascular invasion; or multiple, one lobe, >2 cm, with/without vascular invasion
T4	Multiple, more than one lobe; invasion of major branch of portal or hepatic vein; invasion of adjacent organs other than gallbladder; or perforation of visceral peritoneum
N	Regional lymph nodes
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastases
N1	Regional lymph node metastases
M	Distant metastasis
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis
Stage grouping	
Stage I	T1N0M0
Stage II	T2N0M0
Stage IIIA	T3N0M0
Stage IIIB	T1N1M0, T2N1M0, T3N1M0
Stage IVA	T4, any N, M0
Stage IVB	Any T, any N, M1

must simultaneously maximize the number of viable candidates while at the same time rejecting the smallest number who could have benefited from this treatment. Unfortunately, the current pTNM system (Table 1) has not proven to be predictive of tumor-free survival *(30–32)*. In addition to the shortage of organs, the waiting time for LT is sufficiently long that many patients suffer disease progression and become unsuitable for transplant while awaiting LT. The United Network for Organ Sharing (UNOS), which uses an alter-

Table 2
American Liver Tumor Study Group Modified TNM Staging Classification

T0,N0,M0	Not found
T1	One nodule <1.9 cm
T2	One nodule 2.0–5.0 cm; two or three nodules, all <3.0 cm
T3	One nodule >5.0 cm; two or three nodules, at least one >3.0 cm
T4a	Four or more nodules, any size
T4b	T2, T3, or T4a plus gross intrahepatic portal or hepatic vein involvement as indicated by CT, MRI, or US
N1	Regional (portal hepatitis) nodes involved
M1	Metastatic disease, including extrahepatic portal or hepatic vein involvement
Stage I	T1
Stage II	T2
Stage III	T3
Stage IVA1	T4a
Stage IVA2	T4b
Stage IVB	Any N1, any M1

American Liver Tumor Study Group—A Randomized Prospective Multi-institutional Trial of Orthotopic Liver Transplantation or Partial Hepatic Resection with or without Adjuvant Chemotherapy for Hepatocellular Carcinoma. Investigators Booklet and Protocol 1998.

native staging system based on the American Liver Tumor Study Group (Table 2), currently allows patients with Stage II disease who meet the following criteria to be upgraded on the transplant candidate waiting list in an effort to shorten their waiting time:

> Candidates with Stage II HCC in accordance with the modified Tumor-Node-Metastasis (TNM) Staging Classification that meet all of the specified medical criteria may receive extra priority on the waiting list as specified below. A candidate with an HCC tumor that is ≥2 cm and <5 cm or no more than 3 lesions, the largest being <3 cm in size (Stage T2 tumors) may be registered at a MELD/PELD score equivalent to a 15% probability of candidate death within 3 months.
>
> The candidate must have undergone a thorough assessment to evaluate the number and size of tumors and to rule out any extrahepatic spread and/or macrovascular involvement (i.e., portal or hepatic veins). A pre-listing biopsy is not mandatory, but the lesion must meet the following imaging criteria. The assessment of the candidate should include ultrasound of the candidate's liver,

a computerized tomography (CT) or magnetic resonance imaging (MRI) scan of the abdomen that documents the tumors and a CT of the chest that rules out metastatic disease. In addition, the candidate must have at least one of the following: a vascular blush corresponding to the area of suspicion seen on the imaging studies, an alpha-fetoprotein level of >200 ng/ml, an arteriogram confirming a tumor, a biopsy confirming HCC, chemoembolization of the lesion, radio frequency, cryo, or chemical ablation of the lesion. The alpha-fetoprotein level is required for all HCC exception applications. Candidates with chronic liver disease who have a rising alpha-fetoprotein level $\geq$ 500 nanograms may be listed with a MELD/PELD score equivalent to an 8% mortality risk without regional review board review even though there is no evidence of a tumor based on imaging studies. The candidate cannot be a resection candidate.

Candidates will receive additional MELD/PELD points equivalent to a 10% increase in candidate mortality to be assigned every 3 months until these candidates receive a transplant or are determined to be unsuitable for transplantation based on progression of their HCC. To receive the additional points at 3-month intervals, the transplant program must re-submit an HCC MELD/PELD score exception application with an updated narrative every three months. Continued documentation of the tumor via repeat CT or MRI is required every three months for the candidate to receive the additional 10% mortality points while waiting. Invasive studies such as biopsies or ablative procedures and repeated chest CTs are not required after the initial upgrade request is approved to maintain the candidate's HCC priority scores. Candidates meeting criteria based on an alpha-fetoprotein level of $\geq$500 nanograms must continue to demonstrate an ongoing rise in the alpha-fetoprotein level in order to extend the application.

If the number of tumors that can be documented at the time of extension is less than upon initial application or prior extension, the type of ablative therapy must be specified on the extension application. Candidates whose tumors have been ablated after previously meeting the criteria for additional MELD/PELD points, will continue to receive additional MELD/PELD points (equivalent to a 10% increase in candidate mortality) every 3 months without review, even if the estimated size of residual viable tumor falls below Stage T2 criteria. For candidates whose tumors have been resected since the initial HCC application or prior extension, the extension application must receive prospective review by the applicable RRB.

A candidate not meeting the above criteria may continue to be considered a liver transplant candidate in accordance with each center's own specific policy or philosophy, but the candidate must be listed at the calculated MELD/PELD score with no additional priority given because of the HCC diagnosis. Candidates with HCC including those with downsized tumors (i.e. having undergone ablative therapy) whose original/presenting tumor was greater than a Stage T2), must be referred to the applicable regional review board for prospective review.

5. MOLECULAR PROFILING OF HCC

The explosion of genetic information has impacted liver cancer diagnostics as it has other forms of human cancer *(33–34)*. While a complete understanding of cancer-related gene damage and its effect upon myriad pathways of growth regulation still awaits characterization, the role of cumulative mutational change driving liver cancer development and progression is well established as is the role that such change plays in determining survival and response to different forms of treatment *(35–36)*. Microscopic evaluation alone is a useful, albeit imperfect, tool when used as the sole means to stage and predict tumor behavior.

In order for molecular analysis to be effectively integrated into microscopic analysis, molecular techniques must complement and not compete with microscopic evaluation. Ideally, molecular analysis should take advantage of insights derived from the microscopic examination such as defining the most aggressive sites of cancer growth (i.e., vascular invasion). DNA mutational analysis carried out at different microscopic sites in individual cases of hepatocellular carcinoma demonstrates that molecular heterogeneity is a fundamental property of cancer growth *(37–40)*. This heterogeneity is important as it determines the genotype of the most aggressive neoplastic clone of cells accounting for cancer spread and most in need of effective treatment (Fig. 1).

Slide-based formats for integrated histologic/molecular analyses such as immunohistochemistry (IHC) and in situ hybridization (ISH) are attractive as they retain the histologic format that pathologists are most comfortable examining. IHC and ISH can provide valuable information in liver cancer, especially with respect to gene and protein expression capable of influencing diagnosis and treatment planning *(41–42)*. Slide-based techniques are limited, however, when searching for a mutational change which requires sample manipulation not easily performed on tissue sections. An alternative approach specifically designed to complement microscopic analysis involves microdissection of critical tissue areas followed by mutational analysis in vitro *(43–48)*.

It is well known that HCC's of similar type and histology can show quite variable outcomes *(49)*. There has always been a challenge, from a microscopic perspective, to explain why patients with similar histologic appearing cancers and identical stages of tumor spread can pursue widely divergent clinical courses. Genetic analysis can provide information causally related to biological behavior and treatment responsiveness *(50–51)*. Moreover, mutation acquisition generally precedes and is causally responsible for clinical expression of liver cancer.

A fundamental property of liver cancer is clonal evolution wherein successive clones of phenotypically more aggressive cells overgrow and replace

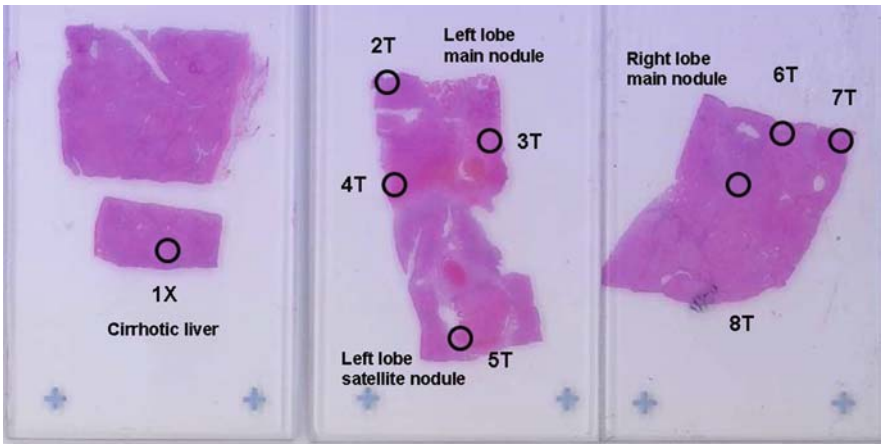

Fig. 1. Selection of microdissection targets for broad panel mutational profiling. Microdissection target selection of multinodular liver cancer. Using standard 4 μm thick recut tissue sections, microscopic features are used to select the best areas of tissue for comparative mutational analysis. Cirrhotic liver can be used as a source of DNA to determine marker informativeness. The remaining targets, the positions of which are shown *dark circles* with their abbreviated designation as described in Table 1, serve as representative targets of the liver cancer. Multiple targets serve as the means to account for intratumoral heterogeneity. Hematoxylin–eosin ×4.

precursor neoplastic cell populations *(52–53)*. This property can, at times, be detected by microscopic observation of increasing cellular anaplasia within an enlarging primary cancer deposit or between primary and metastatic cancer sites. In most instances, however, clonal evolution and its characteristics remain hidden at the microscopic level. It has been shown that clonal expansion is directly correlated with somatically acquired mutational damage leading to progressively greater growth deregulation and metastatic properties (e.g., vascular invasion) *(20–21)*. This is particularly true of liver cancer which has been demonstrated in clinical, animal, and cell culture studies *(20–21)*. Characterizing such temporal changes in tissue specimens obtained at a single point in time requires an appreciation of the admixture of precursor and newly transformed cancer cells in a clinical specimen. This cannot be easily appreciated by relying solely upon the microscopic characteristics of tumor cell shape. Rather an integrated molecular strategy is essential in incorporating a broad analysis of genetic alterations carried out at not one but multiple microscopic sites reflecting topographic and cellular histopathologic features *(5–8)*.

There has been enormous enthusiasm for genome wide methods to diagnose and characterize liver cancer. These methods include comparative genomic hybridization *(54–56)*, RNA expression chip arrays *(57–60)*, and

proteomic analysis *(61–63)*. Each of these techniques is capable of delivering large amounts of highly detailed information concerning the DNA, RNA, and protein content and structure of individual specimens. As tools of discovery, their value has been repeatedly proven. Their application to clinical specimens, however, has been more difficult, related to the obligate need for relatively large amounts of highly purified and good quality tissue for molecular analysis. The reality of small specimen size, optimal tissue fixation, and preservation of correlative microscopic features has generally precluded the broad clinical application of these genome wide methods at this time. Continuing efforts to overcome these obstacles provide hope that they will, at least in part, lead to greater applicability.

Herein we present our experience with microdissection-guided genotyping which is a platform technology to accomplish the goal of integrated microscopic pathology and molecular analysis of routine organ and tissue-based specimens *(11–16)*. The approach is simple, taking the form of two sequential steps. First, cellular specimens undergo optimal fixation and handling leading to detailed microscopic analysis according to established principles. The first step culminates in a (1) microscopic diagnosis and histopathologic characterization and (2) designation of a series of highly representative microscopic tissue targets to be precisely removed to serve as the basis for detailed mutational analysis (Fig. 1). The second step consists of broad panel genotyping for a wide array of mutational markers performed in a high throughput and quantitative manner. A broad panel approach acknowledges the existence of multiple pathways that can lead to liver cancer.

6. MICRODISSECTION-GUIDED BROAD PANEL MUTATIONAL ANALYSIS

Mutational analysis is directed at not one but a broad panel of potential mutational markers (Table 3). This is based on evidence that cancer is related to damage to multiple, potentially interacting, genes leading to overall growth deregulation. The performance of each marker is individually validated to provide discriminating information with the cumulative load of acquired mutational change representing the effects of multiple pathway aberrations on cancer phenotype. The multiparameter approach acknowledges that specific forms of cancer such as hepatocellular carcinoma are not dependent on any single mutation or pathway derangement but involve multiple changes affecting a range of different growth regulatory pathways. In fact, the biological and clinical variability that is evident between different patients with the identical microscopic form of cancer is likely determined, in large part, on the specific constellation of mutated genes leading to uniquely altered pathways of growth control. This is especially true for

Table 3
Microdissection-Guided Mutational Genotyping of Individual Patient Liver Cancer

	CIRR	LT LOBE	LT LOBE	LT LOBE	LT LOBE	RT LOBE	RT LOBE	RT LOBE
	LIV	NOD	MAIN NOD	NOD	SAT NOD	NOD	NOD	NOD
	1N	2T	3T	4T	5T	6T	7T	8T
1p	I	63%	72%	65%	82%			
3p	I					60%	57%	55%
5q	I	57%	52%	53%	77%			
9p	I			57%	63%			
10q	I				60%	86%	92%	93%
17p	I							
17q	I					95%	94%	97%
18q	I	83%	86%	85%	94%			
21q	I	77%	69%	78%	85%	57%	50%	50%
22q	I							

In addition to the cirrhotic liver taken as an internal source of non-cancerous tissue, seven microdissection targets were sampled with each undergoing allelic imbalance (LOH) mutational analysis at ten separate genomic loci. When mutations were found, their presence is indicated by coloration, red or blue, which is used to indicate one or the other of the allele copies. The importance of this designation is that comparison allows a particular neoplastic clone of tumor cells to be tracked in different sites of cancer given that clonality reflects persistence of the same mutation. Furthermore, LOH is measured quantitatively according to the degree of clonality. The greater the number of microdissected cells in a given site that contains a particular mutation, the larger will be the degree of allelic imbalance. Since cancer progression is unidirectional and irreversible within individual tumor cells, the higher the proportion of cells affected by a particular mutation together with the wider distribution across multiple microdissection targets, the earlier in temporal sequence that particular mutation was acquired. Thus defining the quantitative mutational fingerprint at multiple sites enables the unique development and progression of a particular cancer to be characterized.

HCC where multiple pathways have been shown to be responsible for cancer development and progression *(64–66)*. While it would be desirable to catalogue the full extent of such changes, a subset of genetic alterations is generally adequate to address pertinent clinical questions. A typical representation of data so acquired by microdissection genotyping is shown in Table 3.

Tumor suppressor gene loss, the most common cancer-associated genetic alteration, typically follows a two-step process wherein the two copies, or alleles, of the gene become dysfunctional. The first step is often a DNA sequence alteration affecting important functional sites of gene DNA structure. The second step tends to take the form of genomic deletion of the remaining gene together with DNA on either side *(67–68)*. The availability of DNA markers that distinguish allele copies from each other in proximity to tumor suppressor genes allows one to evaluate samples for

cancer-associated DNA alterations. This type of analysis is referred to as allelic imbalance determination, also known as loss of heterozygosity analysis (LOH) *(11–16)*.

After determining the proportion of cells demonstrating allelic loss for a series of mutated microsatellite markers, it is reasonable to arrange the mutations in a timeline of mutation acquisition *(11–16)*. Given that clonal expansion is a driving force leading to replacement of precursor cells with more phenotypically, growth-advantaged, neoplastic cells, mutations acquired earlier in time would be expected to manifest themselves in a larger proportion of cells at a particular tissue target. Allelic loss mutations taking place later in time would be expected to be present in a proportion of cells equal to or less in number than earlier mutational events (Table 3). By microdissecting the specimen at several points, both the time course and topographic distribution of mutational change can be determined. This provides the dynamic link between static morphologic pathology and molecular genotyping capable of defining a unique time course of mutation acquisition for a specific neoplasm.

The most direct application of microdissection genotyping is in the form of a diagnostic analysis to objectively discriminate cancer recurrence from de novo second primary cancer formation *(69)*. This distinction is based upon the concept that metastatic tumors are likely to retain mutational alterations acquired during early stages of cancer growth at the primary site of formation. This application is ideally formulated to address a common challenge in the evaluation of multifocal liver cancer—whether the process is a single neoplasm with intrahepatic metastasis *or* whether it represents two or more unrelated primary malignancies. The genotyping format, using competitive PCR amplification of polymorphic microsatellites situated in proximity to known tumor suppressor genes, enables individual alleles to be detected and quantified. Thus different independent allelic loss mutations for the same microsatellite marker can be detected (Table 3). This extends the concordance analysis of mutational change not just to the specific markers but to specific individual alleles. Finally, the timeline of sequential mutation can be determined based on the degree of allelic loss with earlier events represented by higher degrees of allelic loss. When this is combined with topographic distribution of mutational fingerprinting incorporating the temporal profile of mutation acquisition, this approach allows highly accurate and objective discrimination of tumor recurrence/metastasis versus de novo cancer formation.

Discrimination between new primary liver cancer formation versus intrahepatic spread of cancer is shown in Figs. 1 and 2 and Table 3. Histologically equivalent appearing liver cancer was present in the left and right lobes with the left lobe cancer having a small satellite nodule. Microdissection targets were taken from the peripheral edge of each tumor deposit, with

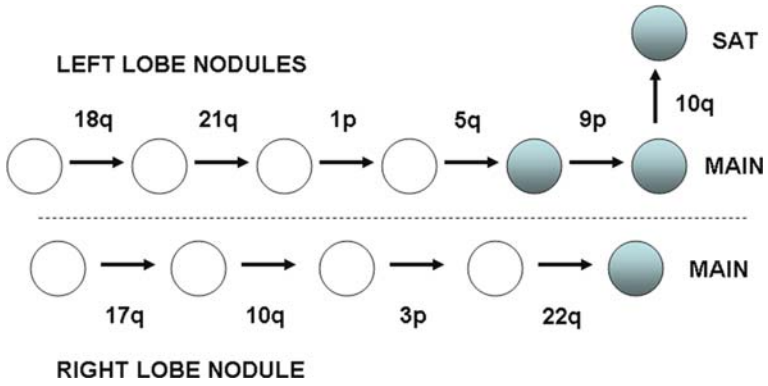

Fig. 2. Schematic representation of temporal sequence of mutation acquisition. Schematic progression model for an individual patient's liver cancer based on microdissection guided mutational profiling. Discordant mutation profiles between the left lobe and right lobe cancer deposits affirms that the two cancers, while histologically similar in appearance, are in fact independent primary malignancies. Each therefore is given its own unique temporal acquisition of mutation schematic. In the case of the left lobe nodules, the concordance in mutational profile and temporal sequence of mutation acquisition affirms that the satellite nodule represents spread of cancer from the left lobe main nodule. However, the satellite contains 9p LOH which establishes its origin from one of the specific targets in the main nodule and the satellite contains an additional 10q LOH mutation. *Shaded circles* represent the mutational profiles of liver cancer that exist in the patient at the time of analysis. *Empty circles* indicate precursor cells during early stages of neoplastic development and progression.

the main nodular deposits undergoing sampling at three separate locations. Mutational changes are summarized in Table 3 where the unique profile of acquired mutations is quantitatively expressed according to each unique microdissection target. In addition to documenting the presence or absence of LOH mutational change at each genomic locus, the specific allele copy affected by LOH (maternal versus parental allele) is indicated in color and the quantitative extent of allelic imbalance is noted.

The independent nature of the left and right liver cancer nodules is readily apparent as there are no shared mutations. Most notably, LOH at 21q is present in both cancer deposits; however, the allele copies affected by LOH are not the same; therefore, this is not the same neoplasm. This represents a powerful use of integrated molecular pathology analysis to definitely and objectively differentiate between one cancer with metastasis versus two independent malignancies. Comparing the left lobe satellite nodule to the adjacent main tumor mass in the left lobe, three mutations are shared between them (1p, 18q, and 21q LOH) involving the same (concordant) allele copies (same color designation). The satellite nodule had acquired 9p LOH that was not present in any of the microdissection targets of the left

lobe main tumor mass. Similarly the three microdissection targets selected to represent the main left lobe cancer nodule lacked evidence of this alteration. Given that neoplastic progression undergoes irreversible forward progression, the most likely accounting is that the satellite nodule is derived from the main left lobe tumor but that it arose from a part of the malignancy represented by microdissection target 4T (Table 1). Comparative mutational profiling performed in this manner enables one to define the precise location from which a metastasis arises.

The use of multiple microdissection targets within the primary tumor shows minor degrees of discordance in keeping with intratumoral heterogeneity (Table 3). The difference represents a minority of later mutational events as expected in a single primary tumor undergoing progressive clonal expansion and growth. Mutations present in the vast majority of clonally expanded liver cancer cells and having been acquired early in temporal acquisition are expected to be present in non-contiguous tumor deposits if the process evolved from a single neoplasm. It is clear that acquisition of additional mutational damage is ongoing in the primary tumor after dissemination of cells originating in a topographically separate site of the tumor. By the same token, new mutational events can be evident in the metastatic deposit that may have occurred following spread to a secondary site. It is quite clear that heterogeneity is a fundamental property of cancer, especially aggressive forms of neoplasia, and that any approach designed to characterize this aspect of tumor biology must acknowledge its existence by multiple site sampling and broad marker analysis.

An interesting observation, consistently displayed by most metastatic neoplasms, is the preservation of the time course of mutation acquisition in metastatic deposits of liver cancer. When HCC is associated with recurrence after a latency interval, there is an identical temporal profile of mutational damage with equivalent proportions of tumor cells bearing specific deletions in primary and metastatic deposits. If metastatic seeding were to have evolved clonally from a single liver cancer cell, then all mutations acquired prior to the seeding event would be expected to be present in all cells of the metastasis. This is in fact not the case but rather the metastasis recapitulates the timeline of mutation acquisition of the primary tumor. This can be accounted for in several ways, but the most likely is metastatic spread not of a single cell but rather of a collection of cells sufficiently large to demonstrate the mixture of remote and recently acquired mutations in the primary tumor. This suggests that an important event in metastatic seeding is the creation of a circulating pool of tumor cell clusters of sufficient size to survive implantation and growth in the metastatic site. It is proposed that these larger clusters possess growth advantages compared to circulating single cells.

Molecular pathology information may be expected to have its greatest impact in prognostication of tumor biological behavior and treatment

responsiveness. This is based on the fact that causally related mutational damage is likely to be responsible for these important attributes of clinical cancer expression. We have found a strong correlation between extent of accumulated mutational change in liver cancer expressed by the factional allelic imbalance rate or FAI (number of total mutations divided by the total number of informative mutational markers in the panel). When the FAI is less than 0.3, indolent biology can be expected, including the low likelihood for the development of metastatic disease. Conversely, when the FAI is greater than 0.3, aggressive biological behavior can be anticipated and managed accordingly *(48)*.

7. LOCOREGIONAL THERAPY FOR HEPATOCELLULAR CARCINOMA: A BRIDGE TO TRANSPLANT

Many patients fall beyond the currently restrictive criteria for liver transplantation. Because of donor graft shortages even patients who meet criteria for listing may have a prolonged waiting time for transplantation. The current UNOS/Milan criteria (Stage 2 shown in Table 2) has been challenged by many as too restrictive, and other expanded criteria have been proposed *(70–71)*. Of paramount concern is the incidence of disease progression while on the transplant waiting list which ranges from 10 to 23% *(72–73)*.

In an effort to confine the tumor to the liver, decrease the tumor mass, and prevent progression to vascular invasion many transplant centers use local regional therapy (LRT) which includes transarterial chemoembolization (TACE), radiofrequency ablation (RFA), and Yttrium-90 (^{90}Y). These approaches, although commonly practiced, lack evidence-based support.

Randomized controlled trials and meta-analyses have established the role of TACE for HCC *(74–77)*. In these trials TACE demonstrated a survival advantage over no treatment (31–63% versus 11–27% at 2 years). However, the utility of TACE as a tool to improve survival after liver transplantation is controversial *(78)*. A case–control series performed by Decaens, comparing 100 patients who underwent TACE prior to liver transplantation with a similar group undergoing transplantation alone, showed that pre-transplant TACE does not influence 5-year survival (59.4% TACE versus 59.3% no-TACE, $p = 0.7$) *(79)*. Oldhafer et al. also showed no difference in survival at 3 years post-transplant if patients received pre-transplant TACE (48% versus 54%). In their series they reported 66% of patients had greater than 50% tumor necrosis following TACE; however, response to therapy did not yield an improvement in survival following transplant *(80–81)*.

One recent report even implies a negative impact of partial necrosis possibly predisposing patients to higher recurrence rates. The theory proposed

is ischemic changes from the embolization may reduce the cellular adhesion of tumor cells and perhaps allow systemic spread at the time of surgery *(82)*. Adachi et al. report that those patients with complete necrosis benefited with improved survival after transplant; however, those patients with only partial necrosis had an increased recurrence rate *(83)*.

An important element of LRT as a bridge to transplant is the utility to downstage patients who are originally outside the UNOS/Milan criteria to a clinical picture that then qualifies for liver transplantation. Interestingly, reports show that those patients down-staged into the UNOS/Milan criteria and subsequently transplanted have the same survival and recurrence rate as patients who present with disease originally within the UNOS/Milan criteria *(84–85)*.

Majno and Bharat each have shown that advanced-staged HCC patients who were down-staged and underwent transplant had a 5-year survival advantage compared to similar patients not treated with TACE *(86)*. However, Graziadei reported that despite tumor down-staging with TACE in their cohort, there was a 30% recurrence rate and less favorable survival compared to those with stage I/II disease (31% versus 94% 5-year survival; $p < 0.001$) *(87)*.

The impact of TACE on disease progression has been explored in multiple reports. The probability of dropout from transplant listing has been reported to be 15% at 6 months *(88)*. Patients without progression of disease while receiving TACE who subsequently underwent transplant have been shown to survive significantly longer than patients who progress while listed and then go on to transplantation *(89)*. Similarly, Otto *(90)* showed that progression of disease after TACE, while awaiting transplant, was a negative predictor of disease-free survival. In both of these series those patients who were outside UNOS/Milan criteria who did not progress while receiving TACE had similar survival to patients who met criteria and went straight to transplant.

When compared to resection for HCC, RFA has a similar survival but a higher rate of local recurrence *(91)*. RFA, when used as a bridge to transplantation, has been associated with complete necrosis in greater than 50% of patients treated for HCC if less than 3 cm *(92–94)*. Lu and Mazzeferro each have shown that the use of RFA as bridge therapy can reduce the dropout rate from the transplant list as compared to patients without treatment *(28–29)*. Pompili reported down-staging nine patients who exceeded UNOS/Milan criteria utilizing RFA and showed that RFA is associated with complete necrosis in 43% of treated patients in their series. Although effective bridge therapy in selected cases, RFA has not demonstrated an improvement in post-transplant survival *(30)*. Based on current reports, RFA has its greatest effect as bridge therapy in patients with tumors $\leq$3 cm who are listed less than 1 year for transplant *(95)*.

Yttrium 90 (^{90}Y) has also been used as regional therapy for unresectable patients with response rates of 39–47% *(96–97)*. ^{90}Y has the potential advantage of less post-embolization syndrome than TACE and less frequent treatments. One report of 35 patients initially with stage III disease showed that the use of ^{90}Y allowed down-staging of 56% of patients, and transplantation was performed in 8 *(98)*. The choice of ^{90}Y or TACE is based on lobar tumor distribution, liver function, portal vein involvement, lung shunting on a Technectium-99 macroaggregated albumin scan, and institutional bias.

Though controversial, resection can be utilized as bridge therapy or as primary therapy with transplantation as salvage after tumor recurrence or liver failure. Multiple series have shown that after resection, 60–80% of patients can still be transplanted without a significant difference in survival *(99–102)*. The strategy of resection before transplant has the potential to save donated liver grafts and decrease the time patients are exposed to immunosuppression; however, this approach requires close and ongoing surveillance. The possibility for recurrence of disease presenting beyond the UNOS/Milan criteria clearly exists. A laparoscopic approach for RFA or resection has the potential to minimize technical difficulties during subsequent liver transplantation and avoid interruption of the enlarged abdominal wall collaterals, preserving as much liver function as possible *(103)*.

Finally, locoregional therapy may act as a biologic test of disease, perhaps allowing patients to remain on the waiting list longer, thereby preventing selection of patients who progress and who would not benefit from LT. Carefully controlled prospective trials are needed to further define the impact and potential effectiveness of LRT as a bridge to liver transplantation.

8. CURRENT RECOMMENDATION FOR TRANSPLANTATION

1. Patients with positive lymph nodes, extrahepatic metastases, and those who cannot be transplanted with negative margins should be excluded from LT.
2. Stage 1 HCC—we believe that patients with Stage 1 disease (who are the most curable) should be transplanted. However, because many expected small tumors turn out not to be HCC on explanted pathology, the current UNOS guidelines do not allow upgrading of Stage 1 tumors on the waiting list, even if the HCC is biopsy proven. If the underlying liver disease of these patients does not generate a sufficiently high MELD to receive an organ in a timely fashion, living liver donation should be considered (so long as the intended recipient is not too ill and there are no other contraindications).
3. Stage II HCC—these patients are entitled to upgrading on the UNOS waiting list and should be transplanted barring other contraindications.

4. Stage III–IVA—currently these patients are not awarded extra points on the UNOS waiting list. We believe that a genotyping analysis could stratify these patients into low and high risk for recurrence. In node negative patients if the lesion(s) and, when present, malignant thrombus show less than 30% FAI, transplant should be considered. However, since these patients are not allowed extra listing points, the patient would have to accept a marginal liver or a living donor.

Some transplant centers have advocated using living liver donors to push the limits of transplantation for HCC (i.e., for those outside the UNOS/Milan criteria). We do not favor this approach. We believe the life of a healthy donor should not be risked for someone with advanced cancer but should, instead, be used for patients with a low risk of recurrence; the limits should be pushed instead with the cadaveric pool of organs saved by using living donors in the low-risk group. Patients who are not expected to receive a transplant in a timely fashion should be offered bridge therapy.

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19 Living Donor Liver Transplantation for Hepatocellular Carcinoma

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CONTENTS

Abbreviation

HCC	hepatocellular carcinoma
LDLT	living donor liver transplantation
DDLT	deceased donor liver transplantation
LT	Liver transplantation
MELD	Model for end-stage liver disease
MHV	middle hepatic vein
UNOS	the United Network for Organ Sharing
A2ALL	the Adult-to-Adult Living Donor Liver Transplantation Cohort Study
AFP	α-fetoprotein

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PIVKA II	protein induced by vitamin K absence factor II
DCP	des-γ-carboxy prothrombin (same as PIVKA II)
UCSF	University of California, San Francisco
TACE	transarterial chemoembolization
RFA	radiofrequency ablation
PEI	percutaneous ethanol injection

ABSTRACT

Over the last 25 years, liver transplantation has been established as a therapy for end stage liver disease. Liver transplantation also appears to be an ideal treatment for unresectable hepatocellular carcinoma (HCC), since it provides the potential for cure of both the HCC and the underlying liver disease. Although the early results of liver transplantation for HCC were disappointing, emerging Milan criteria in 1996 improved the outcome of liver transplantation for HCC as same as that for the other liver diseases.

Living donor liver transplantation (LDLT) has been developed as an alternative to deceased donor liver transplantation, to improve the organ shortage worldwide. The recent development of LDLT in adults has allowed timely grafting for HCC patients. As a result, LDLT for HCC can achieve an acceptable outcome, which is comparable to the outcome of DDLT for HCC. However, the higher recurrence rates of HCC in LDLT recipients compared to that in DDLT recipients has been shown. It should be verified whether this is contributed to confounding by more advanced disease in LDLT recipients. Recently, the expansion of the criteria for LDLT for HCC has been proposed from many centers, which mostly contain the tumor markers such as AFP and PIVKA-α in addition to the factors of tumor morphology. Validation of the novel criteria, verified and selected from those criteria, will be a major advance in indications for liver transplantation for HCC.

Key Words: Living donor liver transplantation; hepatocellular carcinoma; risk factors; proposed criteria; pretransplant treatment

1. INTRODUCTION

Over the last 25 years, liver transplantation has been established as a durable therapy for all types of end-stage liver disease. Liver transplantation also appears to be an ideal treatment for unresectable hepatocellular carcinoma (HCC), since it provides the potential for cure of both the HCC and the underlying liver disease.

Although HCC was an indication for liver transplantation since the first transplant procedure, the early results of liver transplantation for HCC were disappointing, with 5-year survival less than 40% *(1–3)*. In 1996, Mazzaferro

and his colleagues reported improved results in patients who met the Milan criteria (a tumor <5 cm or no more than three tumors, each no larger than 3 cm); this rekindled enthusiasm for the treatment of HCC with liver transplantation *(4)*.

Furthermore, the recent development of living donor liver transplantation (LDLT) in adults has allowed timely grafting for HCC patients and tentative expansion of the criteria for transplant candidacy in patients with HCC – although such expansion is fraught with controversy.

LDLT has been developed as an alternative to deceased donor liver transplantation (DDLT) applying the same standard such as the Milan or UCSF criteria, which has been used for DDLT originally on the assumption that similar outcome can be achieved. However, this assumption has been challenged with unexpected outcomes of LDLT in certain centers.

In this chapter, we review LDLT for HCC, focusing on overall outcomes, risk factors, and proposed criteria to expand the Milan or UCSF criteria in comparison with DDLT.

2. PERSPECTIVES OF LIVING DONOR LIVER TRANSPLANTATION

2.1. History of Living Donor Liver Transplantation

In the late 1980s, mortality rate among children awaiting transplantation exceeds 25% *(5)*. In this desperate situation, the first LDLT for a small child in Brazil was attempted by Raia in 1987 *(6)*. Although the recipient did not survive, Raia's attempt established the technical feasibility of the procedure. In the same year, the first successful LDLT was reported by Strong in Australia *(7)*. The techniques to perform LDLT in children were refined and established in the Eastern hemisphere by Ozawa *(8)* and in the Western hemisphere by Broelsch *(9)*. Early success led directly to significantly decreased mortality for children awaiting liver transplantation. LDLT from adults, most of whom were the parents of the recipients, to children allowed early recovery of the donors as well as the recipients after transplantation because the left lateral segment graft could offer sufficient liver volume to the small pediatric recipients and leave enough remnant liver, 80% of the entire volume, to the adult donors. Those successful outcomes prompted expanding this procedure to all over the world.

With the success of LDLT for pediatrics, surgeons began to offer the procedure to adult recipients. The first such transplants in adults were performed in Japan, where cadaveric donation was previously nonexistent. Indeed, the Japanese pioneered LDLT in adults with the left hemiliver *(10)*. Soon after, the right hemiliver was used by the Hong Kong group in adults *(11)*. Those procedures were expanded to other Asian centers, where the resource of cadaveric donors was universally scarce. Most centers initially used the left

hemiliver and experienced what became known as small-for-size syndrome, characterized by prolonged cholestasis and coagulopathy, with intractable ascites *(12, 13)*. This syndrome occasionally led to early graft loss at the dawn of the history of LDLT in adults. To overcome this syndrome, the right hemiliver was employed in many centers. While the Hong Kong group preferred to use the right hemiliver with the middle hepatic vein (MHV) *(14)*, most other centers preferred to use the right hemiliver without the MHV. In the latter cases, MHV was left to the donor side to prevent the congestion of Couinaud's segment 4 in the remnant liver. Although this procedure reduced the burden on the donor, reconstruction of the MHV tributaries was required in the recipients *(15, 16)*. Instead of using the right hemiliver, one center employed a dual graft, using two left lobes, from two donors to obtain sufficient graft mass *(17)*.

The initial experiences in the United States with left hemiliver LDLT yielded generally poor results. As a result of occasional patient deaths from small-for-size syndrome, interrupting the wider use of LDLT in adults, many centers began to use the right hemiliver from the donor to provide more actual graft mass for the recipients. The first US series were reported in the late 1990s *(18, 19, 20)*. All reports showed excellent outcome in the recipients comparable to the DDLT and minimal morbidity in the donors. This favorable outcome expanded LDLT in adults in Europe as well as in the United States.

Despite the increasing interest in LDLT, the number of such procedures performed annually in the United States has fallen off since 2002 (Fig. 1) largely for two reasons. First, the implementation of the MELD system in February 2002 diminished the necessity for LDLT in patients especially with

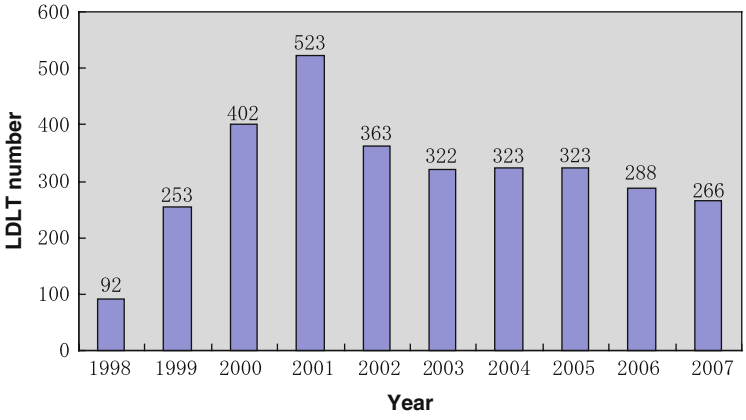

Fig. 1. Annual number of living donor liver transplantation performed from 1998 to 2007 in the United States.

HCC *(21)*. Second, the death of a living donor in January 2002 raised safety and viability questions. After this demise, the United Network for Organ Sharing (UNOS) collects 2-year follow-up data on all donors and is developing standards for evaluating programs as well as resource documents to help standardize the donor consent and evaluation processes. Additionally, a more detailed study of LDLT, the Adult-to-Adult Living Donor Liver Transplantation Cohort Study (A2ALL), a National Institute of Health (NIH)-sponsored multicenter prospective study of LDLT at nine centers in the United States, is underway and recently published excellent outcomes, including a survival benefit for candidates on the waiting list who pursue LDLT.

2.2. Advantages and Disadvantages of Living Donor Liver Transplantation

2.2.1. Advantages

LDLT offers several advantages. First, LDLT can be performed in a timely manner, without a long wait, so that fewer patients are precluded from transplant as a result of disease progression. This factor has become especially helpful for patients who are disadvantaged by the cadaveric organ allocation scheme, including patients with tumors like HCC, cholestatic liver disease, or blood type O, as well as those who are retransplantation candidates.

Second, damage of the liver graft is minimal in LDLT. LDLT offers significantly less ischemic damage to the liver compared to DDLT since the donor and recipient surgeries are elective and performed simultaneously. In addition, liver donors are essentially healthy and the quality of the donated liver is usually superior to the one from cadaveric organs, which can be damaged from the adverse pathophysiologic effects due to brain death.

Third, the indications for transplant can be extended since competition with other candidates for scarce cadaveric organs is eliminated. However, the role of living donor liver transplantation especially for patients with HCC, and the risks to the donor, remains incompletely defined.

Overall, LDLT can increase the potential donor organ pool, conferring a benefit from this life-saving procedure on more people.

2.2.2. Disadvantages

There are a number of disadvantages in LDLT. First, the most important disadvantage is donor mortality and morbidity. Donor safety is the first priority in LDLT. However, the actual risk of death in hepatic lobe donors for LDLT is unknown because of the lack of a comprehensive database. Without the information on the number of donor operations and the known mortality, it is impossible to inform prospective donors of the true risk of the procedure. A recent report identified 33 donor deaths. Deaths in 21 of 33 seem to have been related to the procedure *(22)*. The overall mortality of donation

is estimated to be 0.1–0.3%, possibly reaching 0.5% when using the right hemiliver.

There has been a wide range of complication rates reported in the literature in donors after LDLT. Donor complication rates have ranged from 0 to 100% with a median of 16% *(23)*. Biliary complications and infections are the most commonly reported donor complications. Rates of biliary complication range from 0 to 38.6% with a median of 6.2%, and rates of infection including wound infection, urinary tract infections, and pneumonia range between 1 and 28.6% with a median of 5.8%. From multicenter studies, the donor complication rate is 38% in the A2ALL study and 15.8% in five Asian centers study. Biliary complications are most frequently seen in both studies. The complication rate is higher in the right lobe (28%) than in the left lateral segment (9.3%) or left lobe (7.5%) in the Asian center study.

Second, small-for-size syndrome is the other disadvantage, limiting wider application of LDLT in adults. Enhanced recipient portal hypertension with hyperperfusion of small grafts is thought to be one of the causes of this syndrome *(24)*. A small-for-size graft, as measured by a low graft weight to recipient weight ratio (GW/RW) or by a low graft volume to recipient standard volume ratio (GV/SV), seems to be a major risk factor. A number of studies identified a GW/RW ratio <0.8% or a GV/SV <40% to indicate a significant risk *(13, 25)*. A small-for-size graft, unable to meet the metabolic demands of the recipient, results in the development of small-for-size syndrome, characterized by prolonged cholestasis, prolonged coagulopathy, intractable ascites, and encephalopathy, and often leads to graft failure. Basically the treatment for this syndrome is to reduce the portal flow or pressure. A number of methods have been described including medical interventions such as the use of a somatostatin or a β-blocker *(26, 27)* and surgical interventions such as portocaval shunts, mesocaval shunts, splenic artery ligation, and splenectomy *(28–32)*.

Third, there is a surgical limitation associated with LDLT. The basic principle in performing curative surgery on cancer patients is to resect the tumor, while keeping enough margin between the tumor and the resection line, by removing the vessels and lymph nodes en bloc which might contain direct invasion or metastasis. It is legitimate in liver transplantation for HCC. In cases of DDLT for HCC the entire vena cava as well as the hilar structures, including hepatic artery, portal vein, bile ducts, and lymph nodes, are usually removed, together with native liver. In cases of LDLT however, such technique is not feasible, and the entire native vena cava and the long artery and portal vein are left with the recipients, unless surrogate vascular grafts are available from cadaveric donors. Thus, from the technical point of view, LDLT is less optimal for HCC patients, although one study showed no difference in outcomes when comparing piggyback technique, similar to the

technique of LDLT, to the conventional bicaval technique in DDLT for HCC within the Milan criteria *(33)*.

2.3. Living Donor vs Deceased Donor Liver Transplantation

Early reports failed to demonstrate a convincing advantage of LDLT over DDLT *(34, 35)*. Two studies compared outcomes of LDLT vs DDLT from the UNOS database. Abt reported a higher rate of graft failure in adult LDLT with hazard ratio of (HR, 1.66) compared to DDLT *(34)*. Thuluvath and Yoo found that the 2-year patient survival was similar (79% in LDLT vs 80.7% in DDLT) *(35)*. However, graft survival at 2 years was significantly lower in recipients of LDLT (64.4% vs 73.3%; $p < 0.001$) and the patients who had LDLT were 60% more likely to lose their graft within 2 years compared to DDLT. Those results, however, were heavily influenced by the early experiences of the LDLT program.

The superiority of LDLT over DDLT has been shown with an intention-to-treat study. A single-center study proved that candidates with potential live donors had a lower waiting time mortality compared to candidates without potential live donors, although they were unable to show a significant survival benefit with LDLT over DDLT *(36)*. A similar prospective study also showed that the group with potential live donors had a shorter waiting time for transplantation, greater transplantation rate, lower mortality rate while waiting for a transplant, and better overall survival compared with candidates without potential live donors *(37)*. The advantage of LDLT was confirmed in a recent study. An outcome analysis from the time of listing for 1091 adult patients was reported *(38)*. One hundred and fifty-four patients had a suitable living donor (group 1) and 153 underwent LDLT. Of the remaining patients (group 2; $n = 937$), 350 underwent DDLT; 312 died or dropped off the waiting list; and 275 were still waiting at the time of this analysis. Patients in group 1 had shorter mean waiting times (6.0 months vs 9.8 months; $p < 0.001$) and had a survival advantage from the time of listing (1-year survival 90% vs 80%; $p < 0.001$) compared to patients in group 2.

Those results have been supported by the recent reports from A2ALL *(39)*. Among 807 potential living donor recipients, 389 underwent LDLT, 249 underwent DDLT, 99 died without transplantation, and 70 were awaiting transplantation at last follow-up. Recipients of LDLT showed much lower mortality compared to the candidates who did not undergo LDLT. As centers gained experience with more than 20 cases of LDLT, this benefit was magnified. Post-transplant survival probabilities for LDLT recipients were 84.8% at 3 years compared to 86.3% in DDLT. Post-LDLT survival increased after centers gained experience with more than 20 cases (89.7% at 3 years). LDLT of experienced centers has an advantage over DDLT in intension-to-treat survival as well as post-transplant survival.

3. LIVING DONOR LIVER TRANSPLANTATION FOR HCC

Table 1 presents the demographics and outcomes of 10 single-center *(40–49)* and 3 multicenter studies *(50–52)*. Of ten single centers, eight are from Asia [Japan ($n = 4$), Korea ($n = 2$), China ($n = 1$), and Taiwan ($n = 1$)], two from Europe [Germany ($n = 2$)], and one from the United States. Three multicenter studies were conducted in Korea (4 centers), the United States (9 centers), and Japan (49 centers).

3.1. Indication of Living Donor Liver Transplantation for HCC

The indication for HCC in 10 centers is variable (Table 2). However, all centers uniformly preclude HCC with extrahepatic tumor spread and macrovascular invasion to both portal vein and hepatic vein as basic criteria. Mount Sinai Hospital excludes only the case with invasion to the main portal vein. Table 1 shows the current indication of LDLT for HCC. Most of the centers do not have rigorous criteria and elect the candidates on a case-by-case basis. Five of 11 centers had additional criteria for indication; University of Hong Kong used Milan criteria before 2002 and had expanded their indication to the UCSF criteria (solitary tumor $\leq$ 6.5 cm or $\leq$ 3 nodules with the largest lesion $\leq$ 4.5 cm and total tumor diameter $\leq$ 8 cm). Since 2002, Tokyo University used the criteria of HCC up to five nodules with a maximum tumor size $\leq$ 5 cm in diameter. University Medicine Berlin has been using criteria with no limit in solitary tumor, and maximum tumor size $\leq$ 6 cm and total diameter $\leq$ 15 cm in multiple tumors. Kaohsiung Medical Center follows the Milan criteria strictly.

3.2. Outcomes of Living Donor Liver Transplantation for HCC

3.2.1. Ten Single-Center Studies

LDLT for HCC was performed in 750 patients in 10 centers between 1989 and 2006 *(40–49)* (Table 1). Mean age ranges were from 51 to 58 years, and males were predominant with the ratio ranging from 65 to 91%. Viral infection is associated with background liver disease in most patients. While HBV hepatitis is more predominant in Korea, China, and Taiwan (71–93%), HCV predominates in Western countries and Japan (40–67%). According to the Child-Pugh classification of cirrhosis, 23% (10–40%) are in Child-Pugh class A, 32% (11–47%) in Child-Pugh class B, and 46% (22–56%) in Child-Pugh class C. Overall mortality in 10 centers was 176 (23.5%) with a mean mortality rate of 23.8% (9–42%) and overall recurrence was 130 (17.3%) with a mean recurrence rate of 16.0% (3–27%). A mean median follow-up period in 10 centers was 26 months, with a range from 14 months to 40 months. Mean (range) patient survival rate was 85% (68–98%) at 1 year, 75% (62–96%) at 3 years, and 69% (58–90%) at 5 years.

Table 1
Outcome of Living Donor Liver Transplantation for Hepatocellular Carcinoma

Center	Country	Year	*n*	Liver disease		Pretreatment (%)	Mortality (%)	Recurrence (%)	Patient survival			Median follow-up (months)
				HBV (%)	HCV (%)				1 year	3 years	5 years	
Single-center studies												
Mount Sinai Hospital	United States	2004	36	25	67	33	36	17	75%	60% (2 year)	–	15
Univ. of Hong Kong	China	2007	43	84	14	28*	23	23	97%	80%	58%	33
Kyusyu Univ.	Japan	2007	60	15	77	78	15	22	88%	69%	–	14
Tokyo Univ.	Japan	2007	78	–	62	78	15	8	91%	82%	75%	24
Kyoto Univ.	Japan	2007	136	35	56	74	25	15	–	–	70%	27
Univ. Medicine Berlin	Germany	2007	21	29	57	19	24	14	–	68%	–	23–28 (mean)
Seoul National Univ.	Korea	2007	63	89	5	75	27	27	81%	67%	67%	19

Table 1
(Continued)

Center	Country	Year	*n*	Liver disease		Pretreatment (%)	Mortality (%)	Recurrence (%)	Patient survival			Median follow-up (months)
				HBV (%)	HCV (%)				1 year	3 years	5 years	
Single-center studies (continued)												
Univ. Hospital Essen	Germany	2008	57	–	40	39	42	16	68%	62%	58%	30
Asan Medical Center	Korea	2008	221	93	7	74	22	20	85%	74%	68%	37
Kaohsiung Medical Center	Taiwan	2008	35	71	23	91	9	3	98%	96%	90%	40
Total			750	64	35	65	24	17	85%	75%	69%	27
Multicenter studies												
4 centers	Korea	2005	237	91	5	89	43	14	–	73%	–	26
9 centers (A2ALL)	United States	2007	58	–	60	38	38	29	86%	67%	–	48
49 centers	Japan	2007	653	30	59	71	24	14	83%	73%	69%	21

LDLT: living donor liver transplantation; HCC: hepatocellular carcinoma

*Exact number of pretransplant treatment was not shown (total number of salvage transplantation and TACE)

Table 2
Current and Proposed Criteria for Hepatocellular Carcinoma in Living Donor Liver Transplantation

Center	Country	Year	Basic criteria	Current criteria	Proposed criteria	Within# Milan %	Within# UCSF %	Within # proposed criteria %
Single-center studies								
Mount Sinai Hospital	United States	2004	◯*	(TS > 5 cm not related to worse outcome)		47		
Univ. of Hong Kong	China	2007	◯	Milan criteria, UCSF criteria since 2002		74	84	
Kyusyu Univ.	Japan	2007	◯		TS ≤ 5 cm, PIVKAII ≤300	33 (36)	– (41)	
Tokyo Univ.	Japan	2007	◯	TN ≤ 5, TS ≤ 5 cm		87		92
Kyoto Univ.	Japan	2007	◯		TN ≤ 10, TS ≤ 5 cm, PIVKA II ≤ 400	54		65

Table 2
(Continued)

Center	Country	Year	Basic criteria	Current criteria	Proposed criteria	Within# Milan %	Within# UCSF %	Within # proposed criteria %
Single-center studies (continued)								
Univ. Medicine Berlin	Germany	2007	○	No limit in TS (solitary), TS ≤ 6 cm TTS ≤ 15 cm (multiple)		38	62	
Seoul National Univ.	Korea	2007	○		Scoring system with TS, TN, and AFP	68	79	78
Univ. Hospital Essen	Germany	2008	○		Scoring system with age, MELD, AFP	42	47	
Asan Medical Center	Korea	2008	○		TS ≤ 5 cm, TN ≤ 6, no macrovascular invasion	74	79	84

(*Continued*)

Table 2
(Continued)

Center	Country	Year	Basic criteria	Current criteria	Proposed criteria	Within# Milan %	Within# UCSF %	Within # proposed criteria %
Single-center studies (continued)								
Kaohsiung Medical center	Taiwan	2008	◯	Milan criteria				
Multicenter studies								
4 centers	Korea	2005	◯			71	78	
9 centers (A2ALL)	USA	2007	◯			38	50	
49 centers	Japan	2007	◯		Milan criteria+AFP $\leq$ 200 & PIVKA II $\leq$ 100	54 (61)		77 (81)

LDLT: living donor liver transplantation; HCC: hepatocellular carcinoma;
TN: tumor number; MTS: maximum tumor size; TTS: total tumor size;
AFP: α-fetoprotein; PIVKA II: protein induced by vitamin K absence factor II;
basic criteria: no extrahepatic spread and no vascular invasion (◯*: no invasion of main portal vein);
#: percent within each criterion by pathology (by pretransplant imaging study)

Those outcomes were compared to 2,616 cases of HCC from 19 DDLT centers *(4, 53–71)*. Available overall mortality in 16 DDLT centers was 399 (27.5%) with a mean mortality rate of 26.2% (16–61%), and overall recurrence in 19 DDLT centers was 285 (13.6%) with a mean recurrence rate of 11.3% (3–21%). Mean (range) patient survival rate was 85% (75–94%) at 1 year, 71% (59–79%) at 3 years, and 65% (41–80%) at 5 years. While no significant difference is found in mortality rate between LDLT and DDLT centers, the recurrence rate was significantly worse in LDLT centers compared with DDLT centers (mortality rate: $p = 0.6585$, recurrence rate: $p = 0.0247$).

3.2.2. Multicenter Study in Korea

A retrospective multicenter study revealed the outcomes of 312 HCC patients who underwent liver transplantation at four Korean institutions from 1992 to 2002, through a comparison between a DDLT group ($n = 75$) and a LDLT group ($n = 237$) *(50)* (Table 3). The LDLT group contained more patients with Child A cirrhosis compared with the DDLT group, although overall 3-year survival rate was better in LDLT compared with DDLT (73.2% vs 61.1%: $p = 0.043$). After excluding 38 cases of perioperative mortality, this significant difference disappeared. While HCC recurred in 11 (18%) of 61 discharged DDLT recipients, HCC recurred in 33 (15.5%) of 213 discharged LDLT recipients. Comparison of HCC recurrence curves did not reveal any statistical difference between these two groups. Milan criteria were met in 70.4%: Their 3-year survival rate was 89.9% after DDLT and 91.4% after LDLT with exclusion of perioperative mortality. UCSF criteria were met in 77.7%: Their 3-year survival rate was 88.1% after CDLT and 90.6% after LDLT. The author concluded that the currently available selection criteria, both Milan and UCSF criteria, for patients with HCC can be applicable to LDLT without change of prognostic power in DDLT.

3.2.3. Multicenter Study in the United States

The A2ALL group from nine centers in the United States has studied a total of 106 patients with cirrhosis and HCC who had a potential living donor evaluated between 1998 and 2003 retrospectively *(51)* (Table 3). While most of the characteristics were equivalent between LDLT and DDLT groups, mean AFP level and the percentage of the patients with HCC stage $\geq$ T3 at the time of transplant were higher in LDLT group than in DDLT group ($p = 0.019$, $p = 0.05$), and the percentage of the patients within Milan criteria was lower in LDLT group than in DDLT group ($p = 0.05$). Median waiting time from listing to transplant was much shorter in LDLT group compared with DDLT group ($p < 0.0001$). While 17 (29%) patients had tumor recurrence in LDLT group, none had recurrence in DDLT group. Recurrence was

Table 3
Three Studies of Comparison Between Living and Deceased Donor Liver Transplantation for Hepatocellular Carcinoma

	Center	4 centers, Korea			9 centers, United States (A2ALL)			Univ. Hong Kong, China		
	Year	2005			2007			2007		
	Type	LDLT	DDLT	*p*	LDLT	DDLT	*p*	LDLT	DDLT	*p*
	n	237	75		58	34		43	17	
Child classification	(Child A)	29 (12%)	4 (5%)	0.005				17 (40%)	1 (6%)	0.01
AFP	(ng/ml)				44	13	0.019	33	19	0.131
Tumor stage	(≥T3)	25 (11%)	16 (21%)	0.767	32 (55%)	13 (38%)	0.05			
Milan criteria	(within)	173 (73%)	53 (71%)	0.694	21 (38%)	20 (59%)	0.05	32 (74%)	12 (71%)	
Pretreatment					22 (38%)	11 (32%)	0.590	1 (2%)	4 (24%)	0.001
Median waiting time	(days)				95	373	<0.0001	27	110	0.001
Mortality	*n* (%)	56 (24%)	31 (41%)		22 (38%)	12 (35%)		10 (23%)	1 (6%)	
Recurrence	*n* (%)	33 (14%)	11 (15%)		17 (29%)	0 (0%)		10 (23%)	0 (0%)	
Patient survival	1 year				86.0%	76.0%		97.0%	94.0%	
	3 year	73%	61%	0.043	67.0%	63.0%	0.910	80.0%	94.0%	0.187
	5 year							58.0%	94.0%	
Recurrence	1 year				18.0%	0.0%		7.0%	0.0%	
	3 year	NA	NA	0.884	29.0%	0.0%		29.0%	0.0%	0.029
	5 year								29.0%	0.0%
Follow-up	(months)	26	35	<0.001	48	41	0.039	33	33	NS

LDLT: living donor liver transplantation; DDLT: deceased donor liver transplantation;
AFP: α-fetoprotein
Tumor stage: based on the modification of the TNM staging classification utilized by the Organ Procurement and Transplantation Network

more common in LDLT group at 3 years ($p = .002$). There was no difference in overall mortality between the two groups.

The authors concluded that enthusiasm for LDLT as HCC treatment was dampened by high HCC recurrences compared to DDLT.

3.2.4. Multicenter Study in Japan

A large survey has been conducted from 49 centers of Japan and a total of 653 patients with HCC who received LDLT was reported *(52)*. Median age was 56 years (range, 21–70 years). Males were three times more predominant than females. HCV infection was a leading cause of liver cirrhosis, occurring in 385 recipients (59%) vs HBV for 199 (30%). Half the patients had advanced liver failure with Child C, whereas 30% were with Child B and 10% were with Child A.

Of the 653 recipients, 497 (76.1%) were alive without ($n = 451$) or with ($n = 46$) HCC recurrence; 156 (23.9%) had died of recurrent HCC ($n = 46$) or for other reasons ($n = 110$). A median follow-up period was 21.5 months. Actuarial patient survival was 82.6% at 1 year, 72.6% at 3 years, and 68.9% at 5 years; actuarial disease-free survival was 77.4% at 1 year, 65.1% at 3 years, and 61.5% at 5 years. By univariate analysis, α-fetoprotein (AFP) and protein induced by vitamin K absence factor II (PIVKA II, or DCP, des-γ-carboxy prothrombin), MELD score, and tumor characteristics of explanted livers were found to be important risk factors for patient survival. AFP and PIVKA II/DCP were found to be independent risk factors for patient survival by multivariate analysis.

Ninety-two recipients (14.1%) developed recurrence after LDLT. The cumulative recurrence rate was 9.2% at 1 year, 19.9% at 3 years, and 21.6% at 5 years. Tumor stage, age, AFP, PIVKA II, and pathological characteristics of tumors (e.g., number and size of tumors, distribution, vascular invasion, and differentiation) were closely associated with HCC recurrence by univariate analysis. By multivariate analysis, AFP, PIVKA II, vascular invasion, and number, distribution, and size of tumors were found to be independent risk factors for recurrence

From the fact that high serum AFP and PIVKA II levels before LDLT were closely associated with biological aggressiveness of HCC as expressed by macroscopic vascular invasion, larger tumor size, and more nodules, and related to worse patient survival and disease-free survival, new proposed criteria including AFP and PIVKA II (referred as the A-P level) were introduced. AFP $\leq$200 ng/mL and PIVKA II $\leq$100 mAU/mL were set as cutoff values.

By postoperative pathology study, 5-year disease-free survival of those who met ($n = 325$) and exceeded ($n = 272$) the Milan criteria was 95.3 and 66.4%, respectively. When the A-P levels were below the criteria, the 5-year disease-free survival of the patients who were within and exceeded the

Milan criteria was 99.5 ($n = 208$) and 84.3% ($n = 124$), respectively, while those with higher A-P levels had a 5-year disease-free survival of 85.0% ($n = 96$) and 45.0% ($n = 131$), respectively. Half the patients who exceeded the criteria but who satisfied the A-P levels were found to survive as long as those who met the criteria. Similar results were obtained from a preoperative imaging study.

The authors concluded that by using the Milan criteria and the A-P levels, they could differentiate the outcome of the recipients who were beyond the Milan criteria into two groups: low-A-P level patients (50%) with satisfactory survival without recurrence and high-A-P level patients (50%) with high recurrence rates in both LDLT and DDLT.

3.3. Underestimation and Overestimation of HCC

Accurate diagnosis and staging of HCC is of paramount importance to know the prognosis of the recipients and the eligibility of transplantation which inevitably causes donor risks in LDLT. Many centers are now proposing an expansion of the criteria for consideration of LDLT as well as DDLT. Those proposals are based on recommendations derived from analyses of explant pathology. There is little information on whether the proposed criteria can be accurately defined by preoperative imaging. Since those proposed criteria were initially derived from the explant pathology, it was essential to compare the major tumor features as determined by the pretransplant imaging and the explant pathology before the determination of their clinical applicability. In the series of LDLT, accuracy of HCC stage estimation was examined in two studies. In Seoul National University, accuracy in HCC stage estimation was achieved in 64% of patients, stage underestimation was observed in 13%, and overestimation occurred in 24% *(46)*. In a Japanese multicenter study, Milan criteria diagnosed by imaging studies were concordant with pathological classification in 78.3%, underestimated in 15.8%, and overestimated in 5.8% *(52)*. Underestimation from seven DDLT centers is between 16 and 49% with a mean of 34% *(4, 54, 58, 62, 66, 67)*. Underestimation rate in those two LDLT centers seems to be lower than that of DDLT. Although it is impossible to generalize this to entire LDLT centers, the reason for the lower underestimation rate in LDLT could be explained by improvement of diagnostic radiological imaging techniques because most of the LDLT studies were conducted more recently, compared with DDLT studies. In addition, it is readily conceivable that time elapsed from the last imaging study to the transplantation is usually shorter in LDLT. LDLT was often planned and followed soon after the evaluation with radiological imaging. In contrast, the unpredictable timing of the availability of a deceased donor liver graft makes it difficult to have imaging studies performed immediately before DDLT.

3.4. Risk Factors for Outcome

To identify the risk factors for outcome is important to limit or expand the indication criteria. There were a number of the risk factors in LDLT for HCC, resulting from univariate or multivariate in seven single-center and three multicenter studies (Table 4a, 4b, 4c). Thirteen risk factors were identified to affect the patient outcome independently: age, MELD score, AFP, PIVKA II, tumor size, tumor numbers, tumor differentiation, tumor distribution, vascular invasion (macro- and micro-), UCSF criteria, salvage transplantation, and transplantation in early era.

3.4.1. Tumor Size

Tumor size is the most frequently detected risk factor and is most often included in the indication criteria of liver transplantation. Tumor size is identified in four of six LDLT studies (67%) as an independent factor for recurrence. Most centers set the cutoff value of tumor size at 5 cm. Tumor size is often considered as a surrogate marker of vascular invasion, one of the risk factors most correlated with tumor behavior and recurrence but impossible to be detected preoperatively. There is evidence that the larger the tumor, the more the vascular invasion *(72)*.

3.4.2. Tumor Number

The tumor number is also a commonly detected risk factor and is often included in the indication criteria. The tumor number is identified in three of six LDLT studies (50%) as an independent factor for recurrence. In the series of LDLT for HCC, cutoff value of tumor number was variable; three in most centers, six and ten in each one center. However, tumor number is a relatively weak factor compared to tumor size because it often loses its power to predict the outcome in multivariate analysis even though it is significant in univariate analysis *(73, 74)*.

3.4.3. Tumor Differentiation

The tumor differentiation is identified as a direct index of biologic aggressiveness of HCC *(64, 75, 76)* and is an independent factor for recurrence in one of six LDLT studies (17%). While the tumor differentiation could independently predict the outcome, it is often considered as a surrogate marker of vascular invasion. Some DDLT centers obtained successful outcome, eliminating the patient with poorly differentiated HCC, which was determined preoperatively by percutaneous biopsy *(60)*.

Table 4a
Results of Multivariate Analysis of the Independent Predictor for Patient Survial

Center	Country	Year	*n*	Age	MELD	AFP	PIVKA	TS	TN	Tdiff	Tdist	Macro	Micro	UCSF	Others
Single-center studies															
Univ. Hospital Essen (incl)*	Germany	2008	57	0.005	0.016										
Univ. Hospital Essen (excl)#	Germany	2008	57	0.012		0.0003									
Asan Medical Center	Korea	2008	221					<0.001	<0.001			0.0420			
Multicenter studies															
49 centers	Japan	2007	653			<0.001	0.008								

MELD: model for end-stage liver disease; AFP: α-fetoprotein; PIVKA II: protein induced by vitamin K absence-2;
TS: tumor size; TN: tumor number; T Diff: tumor differentiation; T Dist: tumor distribution;
macro: macrovascular invasion; micro: microvascular invasion;
UCSF: University California San Francisco criteria; (incl)*: including 45-day mortality; (excl)#: excluding 45-day mortality

Table 4b
Results of Multivariate Analysis for the Independent Predictor for Recurrence Free Survival

Center	Country	Year	*n*	Age	MELD	AFP	PIVKA	TS	TN	Tdiff	Tdist	Macro	Micro	UCSF	Others
Single-center studies															
Kyusyu Univ.	Japan	2007	60				< 0.003	< 0.035							
Multicenter studies															
9 centers (A2ALL)	USA	2007	58			< 0.0001									Early Tx (0.0006)

MELD: model for end-stage liver disease; AFP: α-fetoprotein; PIVKA II: protein induced by vitamin K absence-2;
TS: tumor size; TN: tumor number; T Diff: tumor differentiation; T Dist: tumor distribution;
macro: macrovascular invasion; micro: microvascular invasion;
UCSF: University California San Francisco criteria; Early Tx: early transplantation;

Table 4c
Results of Multivariate Ananlysis for the Independent Predictor for Recurrence

Center	Country	Year	*n*	Age	MELD	AFP	PIVKA	TS	TN	Tdiff	Tdist	Macro	Micro	UCSF	Others
Single-center studies															
Mount Sinai Hospital	USA	2004	36								0.02				
Univ. of Hong Kong	China	2007	43											0.010	Salvage Tx (0.047)
Kyoto Univ.	Japan	2007	136				0.0024	0.0341	0.0429						
Asan Medical Center	Korea	2008	221					< 0.001	< 0.001			0.0350			
Multicenter studies															
4 centers	Korea	2005	237					< 0.001		0.012		0.049			
49 centers	Japan	2007	653			< 0.001	< 0.001	< 0.001	< 0.001		< 0.001		< 0.001		

MELD: model for end-stage liver disease; AFP: α-fetoprotein; PIVKA II: protein induced by vitamin K absence-2;
TS: tumor size; TN: tumor number; T Diff: tumor differentiation; T Dist: tumor distribution;
macro: macrovascular invasion; micro: microvascular invasion;
UCSF: University California San Francisco criteria; Salvage Tx: salvage transplantation

3.4.4. Tumor Distribution

The tumor distribution is also a commonly detected risk factor and is identified in two of six LDLT studies (33%) as an independent predictor for recurrence. It is the only one independent predictor in one center *(40)*.

3.4.5. Vascular Invasion

Vascular invasion is a paramount risk factor for HCC patients *(3, 55, 57, 73, 77)* and is identified in three of six LDLT studies (50%) as an independent factor for recurrence. However, this histopathological parameter cannot be used for preoperative selection because it is assessable only by histopathology on the explanted liver. Two surrogate markers have been used to predict the recurrence risk: the histological grade and the tumor size *(72, 78)*. Although both factors are well correlated with vascular invasion, to determine the histological grade percutaneous biopsy is required preoperatively *(60)*.

3.4.6. AFP

AFP is also a commonly detected risk factor, but rarely included in the indication criteria. AFP is identified in one of six LDLT studies as an independent predictor for recurrence (17%). AFP is included in the criteria in three LDLT studies.

3.4.7. PIVKA II/DCP

Prothrombin induced by vitamin K absence factor II (PIVKA II) or des-γ-carboxy prothrombin (DCP) is an immature prothrombin and is a well-recognized tumor marker for its sensitivity and specificity in the screening and diagnosis of HCC *(79)*. PIVKA II is one of the independent predictors for microvascular invasion in the resection of HCC *(80)* and the strongest predisposing factor for the development of portal vein invasion after locoregional therapy for HCC *(81)*. PIVKA II is not a commonly used risk factor worldwide, although extensively measured in Japan, and has been identified as an independent risk factor for recurrence and included in the selection criteria in three studies from Japan.

3.5. Proposed Criteria for Use of LDLT for HCC

There is a major difference between LDLT and DDLT in developing the expanded criteria for HCC. While DDLT centers are expanding the selection criteria, LDLT centers are tightening the selection criteria. Most DDLT centers followed the allocation system based on either Milan criteria or one similar to Milan criteria since its application in 1996. Some DDLT centers have expanded the criteria like UCSF, under the concept that Milan criteria are too stringent. Most LDLT centers, on the other hand, do not have

rigorous criteria in the first place and elect the candidates on a case-by-case basis. Therefore, to make expanded criteria means tightening their criteria for LDLT centers.

Expanded criteria are proposed from five single-center and one multicenter studies (Table 2). Those criteria are based on the independent predictors for outcome derived from the analyses of the pretransplant factors and explant pathology.

The proposed criteria are tumor size $\leq$ 5 cm and PIVKA II $\leq$ 300 in Kyusyu University *(42)*, tumor size $\leq$ 5 cm, tumor number $\leq$ 10, and PIVKA II $\leq$ 400 in Kyoto University *(44)*, tumor size $\leq$ 5 cm, tumor number $\leq$ 6, and no macrovascular invasion in Asan Medical Center *(48)*, and Milan criteria with AFP $\leq$ 200 and PIVKA II $\leq$ 100 in Japanese multicenter study *(52)*. Two centers offer scoring system using three parameters: tumor size, tumor number, and AFP in Seoul National City Hospital *(46)* and age, MELD score, and AFP in University Hospital Essen *(47)*.

To date, the gold standard for selection of HCC patients for both DDLT and LDLT is the Milan criteria, and the UCSF criteria are regarded as acceptable expanded guidelines. Both criteria are based on tumor morphology including tumor size and number. Most of those proposed criteria from LDLT centers include tumor markers such as AFP and PIVKA II in addition to factors of tumor morphology, such as tumor size and number.

The beneficial effect of those proposed criteria can be predicted by the inclusion rates of the patients compared to Milan or UCSF criteria in the same cohort and the outcome of those included patients. While application of the UCSF criteria increases 5–10% of inclusion rates over that of the Milan criteria, those proposed criteria increase 5–26% of inclusion rates over that of Milan criteria (Table 2). Highest inclusion rates over the application of Milan criteria are achieved by the criteria of the Japanese multicenter study. This criterion contains two independent tumor markers, AFP and PIVKA II, which might be the key to achieve the best results.

3.6. Pretransplant Treatment

The rationale for using pretransplant treatment in liver transplantation is (1) to prevent dropouts from waiting list, (2) to improve the post-transplant survival, and (3) downstaging the tumor to meet currently available criteria (ex. Milan) to perform liver transplantation. Thus, in the case of LDLT, the aim of pretreatment is (2) and (3).

A total of 489 patients (70%) in the series of LDLT underwent pretransplant treatment, including transarterial chemoembolization (TACE,49%), radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), and surgical resection (9.9%). While the majority of the patients (74–91%) received pretransplant therapy, less than half the patients in Western

countries (19–39%) did so. No studies described the influence of pretransplant therapy on the outcome, except a Japanese multicenter study in which it was documented that neither pretransplant treatments nor the type of modalities showed any influence on patient survival and recurrence rates *(52)*. An intriguing study was reported by Kyoto University *(82)*. The patients were divided into three groups based on pretransplant therapy: patients without any therapy (group 1), patients with one or two sessions of ablative treatment (group 2), and patients with three or more sessions of treatment (group 3), reflecting differences in the median time elapsed from the diagnosis of HCC to LDLT. The patients who received one or two sessions of ablative therapy had the best 4-year survival (80% for group 2 vs 52% for group 1 and 58% for group 3) and the lowest recurrence rate (9% for group 2 vs 9% for group 1 and 37% for group 3). It seems that one or two sessions of pretransplant treatment brings the best outcome after liver transplantation. This should be confirmed in a further study with a larger cohort.

3.6.1. TACE

TACE is the most popular treatment prior to transplantation. Four case–control studies have been carried out to investigate whether pretransplant TACE improves patient survival after DDLT *(83–86)*. All four studies have failed to demonstrate a beneficial effect of TACE, while tumor necrosis or size reduction (>50%) was achieved in 27–67% of the patients receiving TACE in three studies.

Four prospective studies have been performed to investigate the outcome of downstaging HCC with TACE prior to transplantation. While two studies failed to show the benefit of downstaging with TACE *(87, 88)*, two recent studies have shown improved outcomes *(89, 90)*. Graziadei has compared the effect of preoperative TACE on the outcome of transplanted patients for early-stage HCC and advanced HCC *(87)*. The patients with advanced HCC showed a higher dropout rate from the waiting list (20% vs 0%), higher recurrence rates of HCC (30% vs 2.4%), and worse outcome in the intent-to-treat analysis (31% vs 94% at 5 years, $p < 0.001$) as well as in post-transplantation survival (41% vs 94% at 5 years, $p < 0.001$) compared with patients with early-stage HCC. Pretransplant TACE failed to show benefits in patient survival in advanced HCC. Roayaie has reported another unfavorable long-term outcome of liver transplantation in patients with HCC exceeding 5 cm treated in a multimodality adjuvant protocol including pretransplant TACE *(88)*. The dropout rate was 46% and HCC recurrence rate was 40% in the transplanted patients. Overall and recurrence-free survival rates in transplanted patients at 5 years were 44 and 48%, respectively. Chapman has evaluated outcomes of downstaging patients with advanced HCC (T3 and T4) *(89)*. Eighteen of 76 (23.7%) patients had adequate downstaging to

qualify for OLT under the Milan criteria. Sixteen of 17 (94.1%) patients who underwent OLT are alive at a median of 19.6 months. One patient expired 11 months without recurrence. One patient had recurrent HCC. Yao has reported encouraging results of downstaging of HCC to T2 criteria with pretransplant multimodal treatment, mainly TACE and RFA *(90)*. Eligibility criteria for downstaging included the following: (1) one lesion >5 cm and up to 8 cm; (2) two to three lesions with at least one lesion >3 cm and not exceeding 5 cm, with total tumor diameter up to 8 cm; or (3) four to five lesions with none >3 cm, with total tumor diameter up to 8 cm. Tumor downstaging was successful in 43 patients (70.5%). Thirty-five patients (57.4%) had received OLT, and the intention-to-treat survival at 1 and 4 years after downstaging were 87.5 and 69.3%, respectively. The 1- and 4-year post-transplant survival rates were 96.2 and 92.1%, respectively. No patient experienced HCC recurrence.

3.6.2. RFA

RFA has emerged as the first-line treatment for small, nonresectable cases of HCC because of its high tumoricidal efficacy. There have been only uncontrolled reports about RFA as waiting list treatment. Mazzaferro et al. prospectively treated 60 HCCs in 50 patients meeting the Milan criteria with percutaneous (58%), laparoscopic (36%), or open (6%) RFA *(91)*. All patients were transplanted with a median waiting time of 9.5 months. Complete tumor necrosis was found in 55% of explants. At a median 22 months follow-up HCC recurrence was observed in 4%, and 1- and 3-year survival rates were 95 and 83%, respectively. Lu et al. *(16)* reported on 52 patients who underwent RFA while awaiting LT, with an 11.5% dropout rate at a mean waiting time of 12.5 months *(92)*. Among patients who underwent LT pathologic complete response was found in 65% of the tumors (vs 85% radiologic complete response); with HCC $\leq$3 cm complete pathologic response was achieved in 80%. At a mean follow-up of 14.9 months, there were no HCC recurrences. Both reports support that RFA is a safe and effective bridging treatment, although prospective controlled studies are required in the future.

3.6.3. Surgical Resection

Liver resection is the gold standard treatment for small HCC in Child A cirrhosis. In recent years, some authors have postulated the application of liver transplantation even for resectable small HCC in Child A cirrhosis, which leads to a controversy of whether resection or transplantation should be the initial treatment for patients with small HCC in Child A cirrhosis *(93)*. Recent studies comparing liver resection and transplantation for small HCC revealed worse 5-year recurrence-free survival in liver resection (24–40% vs 60–80%) *(93–97)*. Intention-to-treat analysis of liver resection, however,

showed that the proper selection of candidates for resection promotes better results than liver transplantation, in which the results are significantly hampered by the growing incidence of dropouts because of the increasing waiting time *(98)*. Furthermore, the current shortage of deceased donor organs limits the applicability of liver transplantation for HCC. A significant proportion of HCC patients listed for liver transplantation may dropout from the waiting list because of tumor progression, while liver resection is immediately applicable to many candidates. The use of living donor liver transplantation for patients with small HCCs in Child A cirrhosis may not be justified ethically because of the potential risk to the donors. Thus, liver resection first and salvage transplantation for recurrent tumors or liver failure is an alternative strategy that may reduce the use of liver grafts.

It remains controversial whether salvage transplantation for recurrent HCC could produce results similar to primary transplantation. Adam concluded that liver transplantation after liver resection is associated with a higher operative mortality (28.6% vs 2.1%; $p = 0.0008$), risk of tumor recurrence (54% vs 18%; $p = 0.001$), and a lower 5-year survival (41% vs 61%: $p = 0.03$) compared to primary liver transplantation *(99)*. The worse operation outcome was attributed to the technical difficulties in salvage transplantation. Belghiti, on the other hand, found no significant differences in recurrence rates or in short- or long-term survival: 82 vs 82% at 3 years and 59 vs 61% at 5 years *(100)*. Recently, outcome of salvage LDLT for HCC was reported by Hwang, who compared 200 patients who underwent primary LDLT for HCC with 17 patients who underwent salvage LDLT *(101)*. Overall survival rates after salvage LDLT were similar to those after primary LDLT, especially when the extent of recurrence tumor was within the Milan criteria, although bleeding complications occurred more frequently in salvage LDLT. Further prospective study will be required to clarify the benefit of the salvage transplantation on mortality and long-term outcome.

3.7. Ethical Issues

An ethical dilemma in LDLT for HCC is whether the selection criteria should be the same as DDLT. While a deceased donor graft is a scarce resource subject to the allocation system, a living donor liver graft is a gift to a dedicated recipient. While the decision for DDLT is based on a comparison of the outcome of two recipients, the decision for LDLT is on the balance of the risks and benefits for the donor and the recipient. This special relationship between a donor and a recipient can provide a recipient with the opportunity to undergo LDLT even for advanced HCC. Patients with advanced lesions cannot always be considered as having a contraindication. A high probability of tumor recurrence, however, provokes ethical issues concerning risks to the living donor. The present dilemma is that there are

no accepted criteria for patients with tumors outside the conventional criteria. A living donor is not a public resource but is occasionally directed to a certain recipient with advanced HCC. Because of the unique features of LDLT, the indeterminate survival outcome, such as 5-year patient survival rate of 50%, can be justified without critical impairment of donor ethics. There is another ethical dilemma in LDLT for advanced HCC concerning whether retransplantation using a deceased donor graft should be allowed if living donor graft failure occurred, since these patients were not eligible for deceased donor graft allocation in the first place *(102)*.

4. COMPARATIVE STUDIES BETWEEN LIVING AND DECEASED DONOR LIVER TRANSPLANTATION FOR HCC

One of the advantages LDLT for HCC offers is that it can be performed in a timely manner, without a long wait, so that fewer patients are precluded from transplant as a result of disease progression. Still, the role of living donor liver transplantation for patients with HCC, and the risks to the donor, remains incompletely defined. Two decision analyses used the Markov model to compare LDLT and deceased donor liver transplantation (DDLT) for HCC. One of these showed that LDLT for early HCC offered substantial gains in life expectancy with acceptable cost-effectiveness ratios when the waiting list exceeded 7 months *(103)*. The other study demonstrated that LDLT improved life expectancy by 4.5 years compared with DDLT *(104)*.

The superiority of LDLT over DDLT was confirmed with an intention-to-treat study involving 51 patients with unresectable HCC *(102)*. Twenty-five patients (49%) had voluntary living donors (group 1) and 26 did not (group 2). Four living donors were not suitable for transplantation; the remaining 21 patients in group 1 underwent LDLT after a median waiting time of 24 days (range, 2–126 days). Of the 30 patients who remained on the waiting list for DDLT, only 6 underwent DDLT after a median waiting time of 344 days (range, 22–1359 days, $p < 0.005$). The 1- and 4-year intention-to-treat survival rates were 88 and 66%, respectively, for group 1 and 72 and 31%, respectively, for group 2. The authors concluded that LDLT allowed more patients to undergo early transplantation and resulted in better outcomes.

Three studies compared post-transplant outcomes of patients with HCC between LDLT and DDLT *(41, 50, 51)* (Table 3). Two multicenter studies were described above in detail. Two of three studies showed significantly higher HCC recurrence rates in LDLT recipients. In a Korean multicenter study, HCC recurrence rates were similar between the LDLT and DDLT groups (15.5 and 18%, respectively) *(50)*. The demographics showed that

the patients seemed equally distributed between LDLT and DDLT groups, in both tumor stage and Milan criteria, although more patients with Child A were included in the LDLT group.

The A2ALL study has demonstrated that recurrence was more common in LDLT at 3 years (29% vs 0%: $p = 0.002$) *(51)*. This might be attributed to the selection bias as shown in the demographics that more patients with advanced HCC, AFP level, tumor stage, and Milan criteria were included in LDLT group. The organ allocation system in the United States assigned higher priority to patients with stage 2 HCC. This results in patients within Milan criteria receiving DDLT while patients outside of Milan criteria receiving LDLT. However, selection bias cannot account for everything about the higher rate of HCC recurrence among LDLT recipients. No DDLT patients, including 14 patients with stage T3 or T4, had HCC recurrence. LDLT patients, including 15 patients with stage T1 or T2, had recurrence. Also, the time to recurrence in LDLT recipients was significantly shorter than that of DDLT recipients after adjustment for tumor stage.

The third study by the University of Hong Kong also showed unexpectedly higher recurrence rates in the LDLT group compared with the DDLT group *(41)*. This study looked at outcomes of transplantation in 43 living donor recipients and compared them with the outcomes of 17 deceased donor recipients. All of these patients met Milan or UCSF criteria. The MELD scores, Child-Pugh-Turcotte (CPT) scores, and etiology of liver disease and tumor stage in the explant were comparable in both groups, but there were more patients with Child A or MELD score <10 in the LDLT group. Ten of 40 (25%) patients of the LDLT group underwent salvage transplantation after resection or ablation compared with 1 of 12 (8%) of the patients who received a DDLT. Tumor recurrence developed in 10 of 43 (23%) LDLT patients and none of 17 DDLT patients. Multivariate analysis revealed that salvage transplantation [relative risk (RR), *5.2*] and tumor outside of UCSF criteria (RR, 4.1), but not LDLT, were the only independent predictors of disease recurrence. The authors argued that more patients with salvage transplantation belonged to LDLT group. It is possible that more patients who had tumor with aggressive biological behavior were included in the LDLT group from the fact that more pretransplant recurrences after resection and ablation tended to be rescued by salvage LDLT. Eight of 11 patients who underwent salvage transplantation had microscopic vascular invasion, suggesting that tumor number and size may not be applicable to patients with recurrence after resection or ablation. The authors conclude that the higher recurrence rate seen in LDLT is due to confounding by more advanced disease.

There are several possible explanations why recurrence is higher in the LDLT group compared with DDLT group. It is hypothesized that putting patients with HCC in the fast track to transplant may not provide adequate

time to access the tumor's biological behavior. Inclusion of patients with more aggressive tumors in the LDLT group may account for the higher recurrence rate compared to DDLT recipients who had significantly longer waiting time. Kulik emphasized the importance of a waiting time of 6 months, which might identify patients with slow-growing tumors and those responsive to therapy *(105)*.

Another possible explanation derived from the nature of LDLT, since a graft from a living donor is frequently small for size. Previous animal studies and clinical experience have demonstrated that acute-phase small-for-size graft injury is characterized by hepatic sinusoidal damage that results from excessive portal venous flow and transient portal hypertension *(106, 107)*. The severe shear stress from the portal hemodynamic force triggers a series of inflammatory cascades leading to acute phase graft injury and tumor growth together with cell proliferation, angiogenesis, stellate cell activation, cell signal pathway related to migration and invasion.

Hepatocyte growth factor (HGF) has been suggested to initiate both hepatocyte and tumor cell proliferation after partial hepatectomy. Efinova investigated HGF levels of humans after hepatic resection for HCC and living donor hepatectomy, and demonstrated that HGF plays an important role in hepatocyte proliferation but, contrary to in vitro results, HGF does not seem to play a major role for the progression of hepatoma cells in vivo *(108)*. Hwang revealed the relation between small-for-size grafts and recurrence of HCC within Milan or UCSF criteria after LDLT, and small-for-size graft does not increase the risk of HCC recurrence when HCC is within the criteria *(109)*. Further investigation will be required to confirm whether the small-for-size injury and liver regeneration provoke tumor growth and metastasis.

5. SUMMARY

Despite a wide spectrum of selection criteria among LDLT centers, LDLT for HCC can achieve an acceptable outcome, which is comparable to the outcome of DDLT for HCC. However, the higher recurrence rates of HCC in LDLT recipients compared to that in DDLT recipients have been shown by two studies. Although the most plausible causes are attributed to the selection bias from different indications between LDLT and DDLT, it is essential to obtain more definite conclusions and elucidate all possible causes of recurrence, performing well-designed prospective studies with larger cohorts.

One of the most crucial requirements in liver transplantation for HCC is the advent of expanded criteria which allow more patients with HCC to receive the organs and offer similar or even better results compared to Milan

or UCSF criteria. Apparently many criteria are proposed from LDLT centers, which mostly contain the tumor markers such as AFP and PIVKA II in addition to the factors of tumor morphology. Validation of novel criteria, verified and selected from those criteria, will be a major advance in indications for liver transplantation for HCC.

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20 Medical Therapy of HCC

Brian I. Carr, MD, FRCP, PhD and Srikanth Nagalla, MD, MS

CONTENTS

ABSTRACT

Systemic chemotherapies have been evaluated for many years, with minimal responses and little survival advantage. Regional chemotherapy produces high response rates and two randomized trials also showed survival advantage, using cisplatin or doxorubicin, each plus embolization. ^{90}Yttrium

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also looks promising now. The combination of TACE or ^{90}Yttrium with the anti-angiogenics and cell cycle inhibitors is beginning to be explored.

Key Words: Systemic TACE; Intra-arterial; Cisplatin; RCT; Responses; Survival; Adjuvant

1. PRINCIPLES

1.1. Clinical Presentation

The principles underlying medical management of HCC are based on an understanding of the clinical setting, the tumor characteristics, and the underlying biology. Reviewing our patient population, we found that 81% of patients had cirrhosis and 19% had no evidence of cirrhosis by biopsy or CT scan (Table 1). The male:female ratio was 2.5:1 with 72% of our patients being Caucasian. Interestingly, 24% of our patients had no symptoms at all, but were diagnosed either by the finding of elevated liver function tests on routine physical examination or as an incidental finding, such as a work-up for some unrelated disease. A further 17% of patients were diagnosed because of a planned surveillance CT scan screening because of a known history of hepatitis B or C and/or cirrhosis. About 18% of patients had the symptoms of cirrhosis that included ankle swelling, abdominal bloating, increased girth, pruritis, encephalopathy, or a GI bleed, and a full 40% of patients presented with abdominal pain. This appeared to be the most common presenting symptom in our patient population. We also found that a significant proportion of our patients had weight loss, general malaise or weakness, and loss of appetite. We have recently found that more than 80% of patients report loss of sexual function or desire within the preceding 12 months of the diagnosis (Chapter 24). This appears to be a sensitive but non-specific correlate of our cancer patients and was found on analysis of our systematic study of Quality of Life questionnaires. The tumor characteristics tend to display interesting patterns. In our experience, HCC is typically a multifocal and bilobar tumor (Table 1, tumor characteristics) and is thus often not a surgeon′s disease. In addition, portal vein invasion of either the main portal or main branch portal vein, as judged by occlusion of flow or expansion of the vein on CT scan, occurred in 75% of our patients (Table 2).

1.2. The Underlying Liver Disease

Metastatic cancer that spreads to the liver from organs such as the breast, colon, or lung spread to a normal liver. By contrast, most patients with hepatocellular carcinoma (HCC) typically have a diseased underlying liver as well as the cancer. Although this varies from country to country, between 60 and 90% of HCC patients have underlying cirrhosis *(1)*. The cause of this may vary, but the most common factors are hepatitis B virus (HBV),

Table 1
Clinical Presentation of HCC, University of Pittsburgh, Liver Cancer Center, *n* = 547 (1989–2001)

Symptom	*Patient number*	*(%)*
No symptom	**129**	**(24)**
Abdominal pain	219	(40)
Others (work-up of anemia and various diseases)	64	(12)
Routine physical exam finding, elevated LFTs	129	(24)
Weight loss	112	(20)
Appetite loss	59	(11)
Weakness/malaise	83	(15)
Jaundice	30	(5)
Routine CT scan screening of known cirrhosis	92	(17)
Cirrhosis symptoms (ankle swelling, abdominal bloating, increased girth, pruritis, encephalopathy, GI bleed)	98	(18)
Diarrhea	7	(1)
Tumor rupture	1	
Patient characteristics		
Mean age (years)	56 ± 13	
Male:female	205:1	
Ethnicity (%)		
Caucasian	72	
Middle Eastern	10	
Asian	13	
African American	5	
Cirrhosis	**81%**	
No cirrhosis	**19%**	
Tumor characteristics		
Hepatic tumor numbers		
1	20%	
2	25%	
3 or more	65%	
Portal vein invasion	75%	
Unilobar	25%	
Bilobar	75%	

Table 2
Treatment Options for Hepatocellular Carcinoma

Potentially curative options
Liver resection
Liver transplantation
Other treatments
Regional therapies
(1) Ablative therapies: cytoreductive therapies
Palliative resection
Cryosurgery
Microwave ablation
Ethanol injection
Acetic acid injection
Radiofrequency ablation
(2) Transcatheter hepatic artery treatments
Transarterial chemotherapy
Transarterial embolization
Transarterial chemoembolization
Transarterial radiotherapy
^{90}Yttrium microspheres
^{131}I-Lipiodol
Gene therapies
(3) External beam conformal radiation
(4) Systemic therapies
Chemotherapy
Immunotherapy
Hormonal therapy
Growth factor or antibody control of cell cycle
Supportive palliative care

hepatitis C virus (HCV), chronic alcohol consumption, chronic exposure to mycotoxins, such as aflatoxin B_1 in Africa and Asia, and obesity (NASH) as has been recently appreciated (Chapters 1, 2, 7, 8, and 9). This has major implications for therapy, since the cirrhosis limits the ability of the surgeon to safely resect liver mass without risk of liver failure in the remaining liver, and it limits the ability of the chemotherapist to deliver cytotoxic drugs without risk of liver failure, due to additional damage to the liver that is already damaged due to chronic disease.

1.3. HCC Is a Multifocal Disease

Since HCC typically arises on the basis of cirrhosis, and there are millions of cirrhotic nodules in an individual liver, HCC is often multifocal and bilobar (Table 1, tumor characteristics). Although countries with screening programs are able to diagnose earlier and smaller HCCs, its natural history includes the development of multiple 'satellite' lesions in both lobes of the liver over time. The cause of this is twofold. First, studies with HBV integration sites show that multiple distinct primary tumors can arise in different parts of the liver either synchronously or metachronously. Second, a clonal HCC can spread throughout the liver via portal vein invasion or arterial–venous connections. In addition, the evidence from liver transplant indicates that HCC is commonly a whole organ disease.

1.4. HCC Is a Vascular Tumor

A characteristic of HCC, which distinguishes it from most metastases to the liver, is that it is a highly vascular tumor. This is typically found on the arterial phase of triple-phase helical CAT scans (Fig. 1) or on hepatic angiography (Figs. 2, 3, 4, and 5). This is in contrast to metastases from colon cancer, which are typically hypovascular. This vascularity provides an opportunity for selective delivery of drugs to the tumor, since the vascular

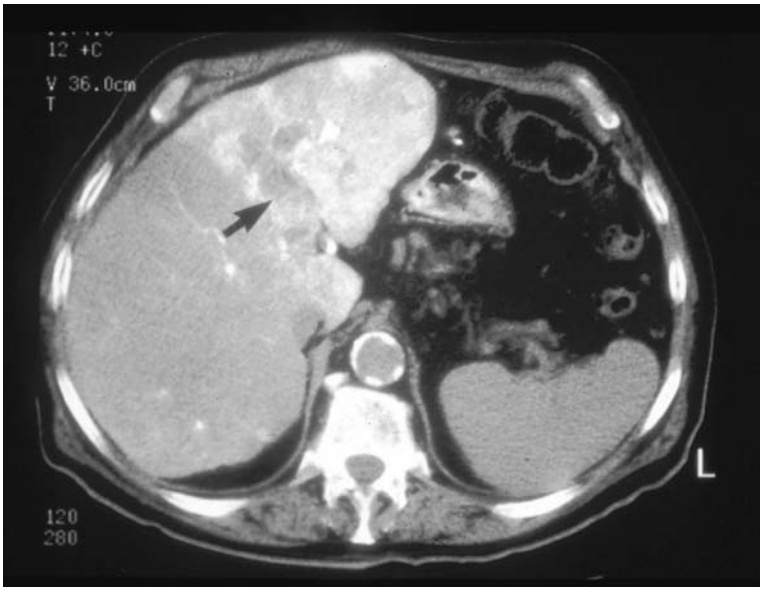

Fig. 1. CAT scan of liver showing a vascular HCC and portal vein thrombus *(arrow)*.

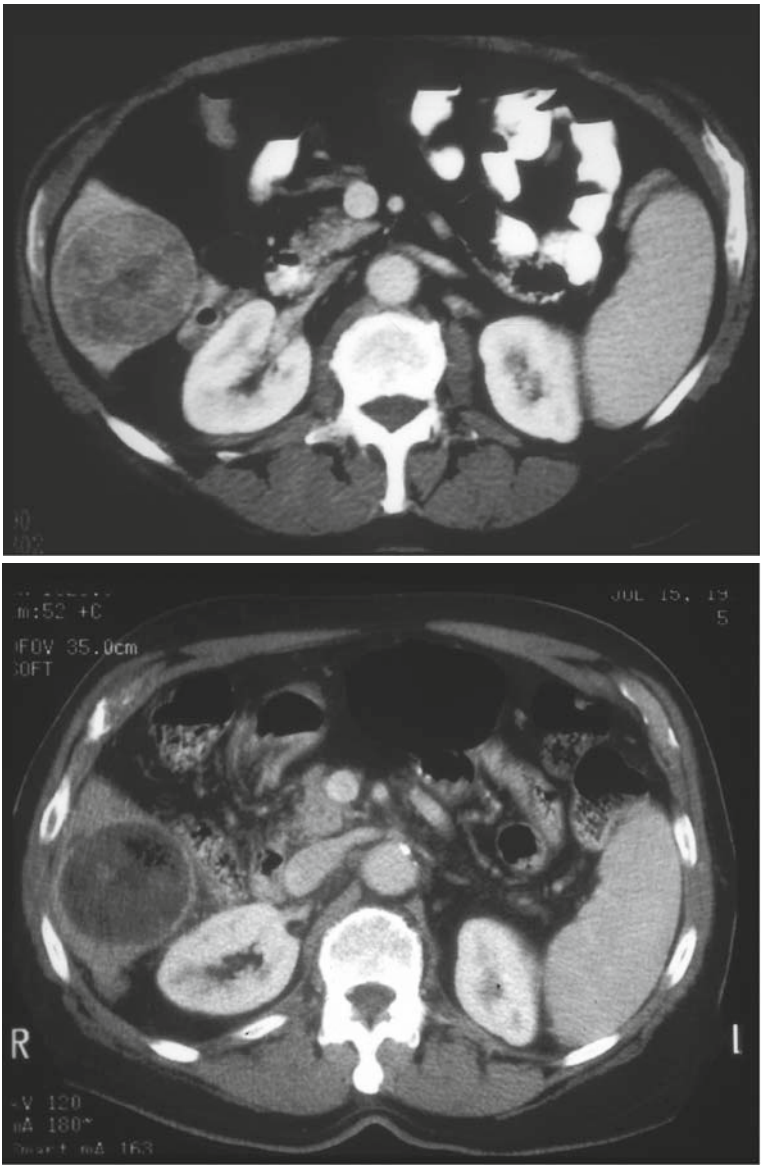

Fig. 2. CAT scans showing a change in tumor vascularity without size change, as a result of chemoembolization.

supply to HCC typically arises from hepatic arteries, whereas the delivery of 90% of the oxygenated blood to the underlying non-tumorous liver is mainly from the portal vein. This provides a partial basis for intrahepatic chemoembolization or intrahepatic chemotherapy, which permits a relatively

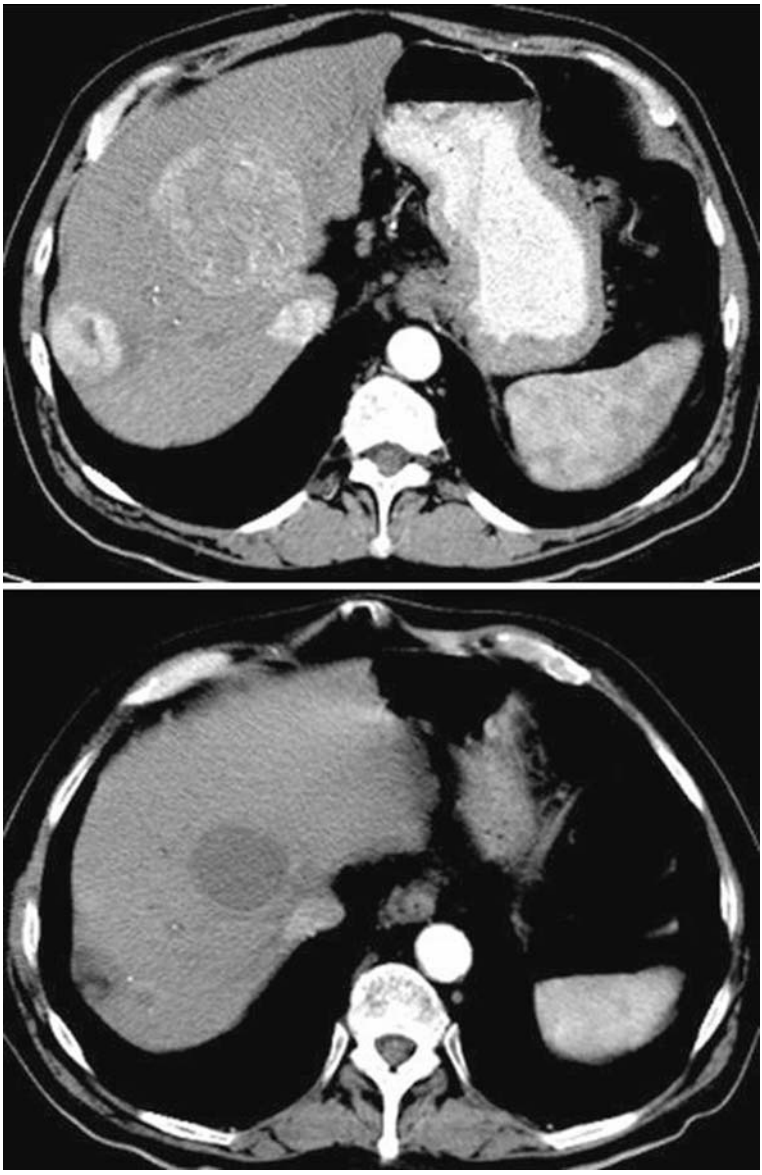

Fig. 3. CAT scans showing change of vascularity and size, as a result of chemoembolization.

selective delivery of chemotherapy to the tumors in the liver via the tumor neo-vasculature that typically grows in response to the presence of an HCC. The other reason is that vascular slowing leads to an increase in hepatic dwell time of infused chemotherapy.

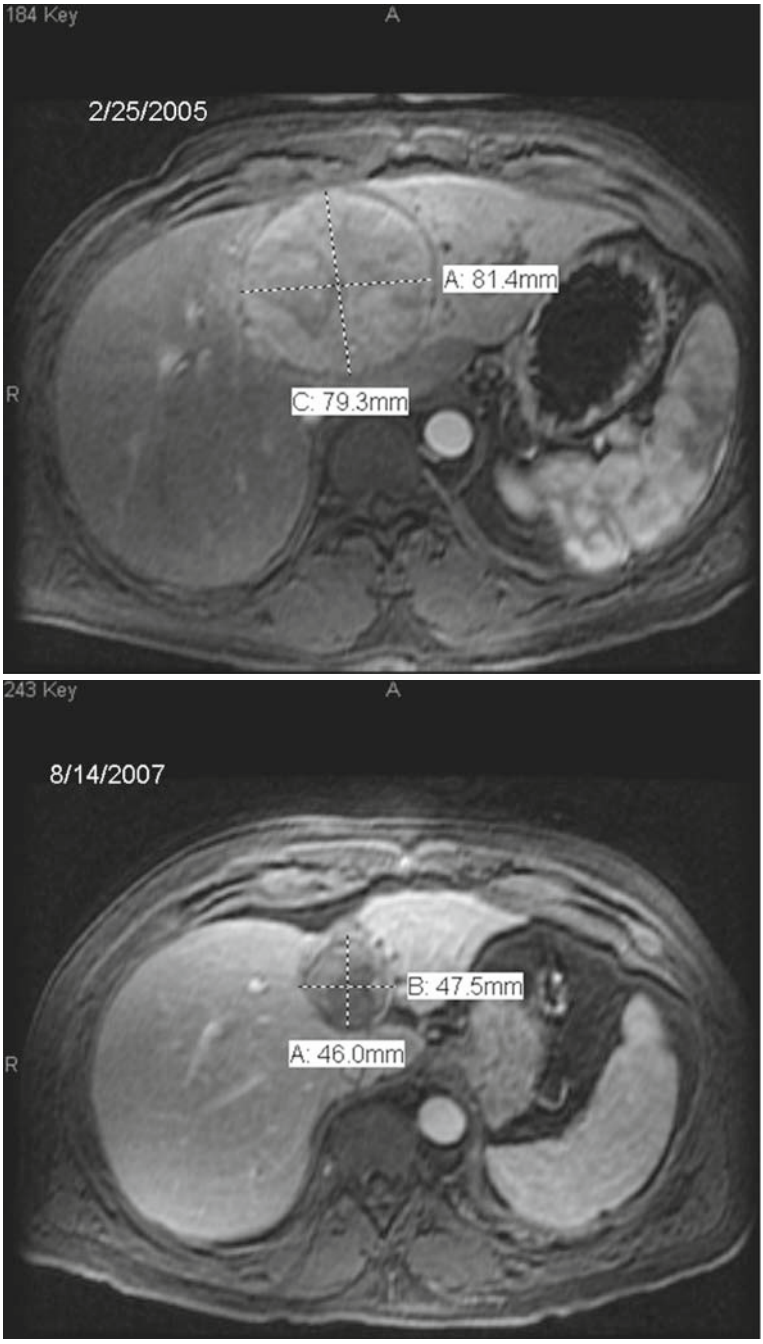

Fig. 4. CAT scans showing change of vascularity and size, as a result of chemoembolization.

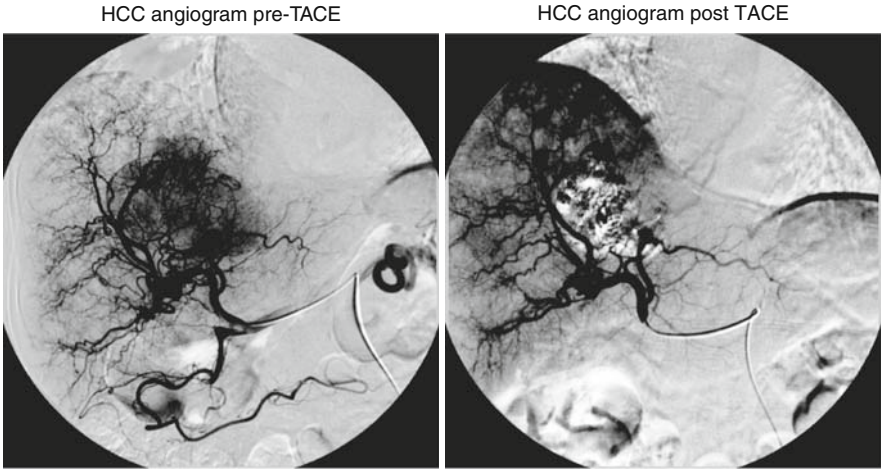

Fig. 5. Angiogram showing vascular changes as a result of chemoembolization.

1.5. Portal Vein Invasion: A Key Prognostic Characteristic of HCC

The tendency of HCC to invade the portal vein is a characteristic of HCC and distinguishes it from most metastases to the liver. It is manifested clinically as thrombosis of a major portal vein or a major portal vein branch (Fig. 1) seen as occlusion and/or expansion of the portal vein on CAT scan or microscopically as presence of HCC in the walls or lumens of normal hepatic vessels. It is also probably the most important negative prognostic factor in the evaluation of the HCC patient for any form of surgery, but particularly for liver transplant. Since the portal vein is thrombosed, it can be safely biopsied by a percutaneous needle and this provides proof for the malignant nature of portal vein thrombosis in the presence of HCC *(2, 3)*. It is currently deemed to be a major contraindication for liver transplant. Portal vein thrombosis has previously been thought to be a contraindication for hepatic artery chemotherapy, because if the portal vein is blocked by tumor and the hepatic artery is embolized for therapeutic purposes, then that lobe of the liver is thought to undergo necrosis, with resultant liver failure. However, as shown below, most of our patients with advanced HCC have portal vein thrombosis, at least of a major branch, and most of them are unresectable. Despite this, most of them have been treated with intrahepatic chemo-occlusion with little deleterious effect on the underlying liver, provided certain precautions are observed (below). These include treating only one lobe of the liver at any single chemotherapy session, as well as using sub-occlusion but never complete embolization of the treated hepatic artery.

1.6. HCC Is Relatively Resistant to the Toxic Effects of Most Chemotherapeutic Agents

It has been known for more than 70 years since the experiments of Haddow *(4)* that the liver that has been damaged by carcinogenic or other toxic chemicals and which then recovers becomes remarkably resistant to a subsequent challenge by a variety of toxic agents *(5)*. Most other cancers such
as breast cancer adapt to chemotherapy by developing 'acquired resistance' to the toxic effects of the chemotherapy. It is thought that most HCC arises ab initio as a drug-resistant tumor. This was most clearly demonstrated in the drug resistance/growth inhibition model of rodent carcinogenesis first described by Solt and Farber, but many other studies have shown the carcinogen-altered liver to be remarkably resistant to toxicity by a variety of poisons *(6)* or cancer chemotherapy agents *(7)*. The clinical consequence of this is that most clinical trials of phase 2 and phase 3 chemotherapy drugs have shown responses to single drugs in less than 20% of the patients and have no beneficial effect on survival (Table 3). However, when the same drugs are given by the hepatic artery route, they have been found to result

Table 3
Selected Recent Studies of Chemotherapy

Investigations	*Drug*	*Partial Response Rate (%)*
Systemic chemotherapy		
Sciarrino et al. *(8)*	Doxorubicin	0
Chlebowski et al. *(9)*	Doxorubicin	11
Ihde et al. *(10)*	Doxorubicin	15
Falkson et al. *(11)*	Doxorubicin, 5-fluorouracil,methyl-CCNU	19
Falkson et al. *(12)*	Neocarzinostatin	8
Ravry et al. *(13)*	Doxorubicin, bleomycin	16
Cavalli et al. *(14)*	VP-16	13
Melia et al. *(15)*	VP-16	18
Melia et al. *(16)*	Cisplatin	1
Ravry et al. *(17)*	Cisplatin	0
Falkson et al. *(18)*	Cisplatin	17
Falkson et al. *(18)*	Mitoxantrone	8
Colleoni et al. *(19)*	Mitoxantrone	23

Table 3
(Continued)

Investigations	*Drug*	*Partial Response Rate(%)*
Chao et al. *(20)*	Paclitaxel	0
Patt et al. *(21)*	5-FU + IFN	18
Patt et al. *(22)*	5-FU + IFN + cisplatin + doxorubicin	20
Bobbio-Pallayicini et al. *(23)*	Epirubicin + VP-16	39
Okada et al. *(24)*	Cisplatin, mitoxantrone + 5-FU	33
Guan et al. *(25)*	Gemcitabine	2
Taïeb et al. *(26)*	Gemcitabine, oxaliplatin	19
Lee et al. *(27)*	Doxorubicin, cisplatin	19
Ikeda et al. *(28)*	5-fluorouracil, mitoxantrone, cisplatin	27
Zhu et al. *(29)*	Epirubicin, thalidomide	0
Zhu et al. *(30)*	Gemcitabine, oxaliplatin, bevacizumab	20
Kim et al. *(31)*	Epirubicin, cisplatin, UFT, leucovorin	17
Park et al. *(32)*	Doxorubicin, cisplatin, capecitabine	24
Louafi et al. *(33)*	Gemcitabine, oxaliplatin	18
Li et al. *(34)*	Gemcitabine, oxaliplatin	2
Uhm et al. *(35)*	Oxaliplatin, doxorubicin	16
Asnacios et al. *(36)*	Gemcitabine, oxaliplatin, cetuximab	20
Yeo et al. *(107)*	PIAF, platinum, interferon, adriamycin, fluorouracil	21
Reviews *(37–44)*		

in tumor shrinkage and ‘partial responses’ (PR) in 30–70% of the patients, usually in association with some form of hepatic artery occluding agent (Tables 4 and 7). Hepatic artery occlusion alone does not appear to impact the tumor, as the results of hepatic artery ligation showed long ago. Several recent randomized trials have shown the benefits of TACE in causing tumor shrinkage (partial responses) as seen in Table 5, but only recently have two randomized clinical trials comparing TACE to no therapy as a control arm convincingly shown a survival advantage for TACE therapy (Table 6), using cisplatin *(92)* or doxorubicin *(93)*, respectively.

Table 4
Intrahepatic Artery Chemotherapy for Hepatocellular Carcinoma

Investigation	*Agents*	*Response Rate (%)*
Sasaki et al. *(45)*	Platinum—gelatin sponge	65
Kasugai et al. *(46)*	Platinum—ethiodized oil	38
Ohnishi et al. *(47)*	MMC—microcapsules	32
Lin et al. *(48)*	5-FU—Ivalon	32
Fujimoto et al. *(49)*	5-FU/MMC—starch	68
Audisio et al. *(50)*	MMC + microcapsules	43
Kobayashi et al. *(51)*	Doxorubicin + ethiodized oil	42
Kanematsu et al. *(52)*	Doxurubicin + ethiodized oil	47
Shibata et al. *(53)*	Platinum + ethiodized oil	47
Konno et al. *(54)*	SMANCS + ethiodized oil	90
Pelletier et al. *(55)*	Doxorubicin + gelatin sponge	17
Carr et al. *(56)*	Doxorubicin/cisplatin	50
Venook et al. *(57)*	Doxorubicin/cisplatin/MMC + gelatin sponge	24
Ohnishi et al. *(58)*	MMC + microcapsules	28
Ohnishi et al. *(58)*	MMC + gelatin sponge + microcapsules	57
Beppu et al. *(59)*	Cisplatin + ethiodized oil + aclarubicin microspheres	50
Trinchet et al. *(60)*	Cisplatin + ethiodized oil vs 0	16
Chang et al. *(61)*	Cisplatin + Gelfoam + ethiodized oil vs Gelfoam + ethiodized oil	68* 67*
Stuart et al. *(62)*	Doxorubicin, ethiodized oil + Gelfoam	43
Bruix et al. *(63)*	Gelfoam, no chemotherapy	81

Table 4
(Continued)

Investigation	*Agents*	*Response Rate (%)*
Carr et al. *(64)*	Doxorubicin, cisplatin + Spherex	63
Carr et al. *(65)*	Doxorubicin, cisplatin + ethiodized oil vs doxorubicin	57
	+ cisplatin	47
Carr et al. *(66)*	Cisplatin	58
Ngan et al. *(67)*	Cisplatin, ethiodized oil, Gelfoam	41
Yamamoto et al. *(68)*	IL-2	
Kawai et al. *(69)*	Epirubicin + Gelfoam vs doxorubicin + Gelfoam	*
Yoshimi et al. *(70)*	Resection vs TAE	*
Epstein et al. *(71, 72)*	Cisplatin + hepatic radiation	48
Rougier et al. *(72)*	Doxorubicin + Gelfoam	41
Onohara et al. *(73)*	Cisplatin	55
Kajanti et al. *(74)*	Cisplatin	40
Nagasue et al. *(75)*	Epirubicin	15
Carr et al. *(76)*	Cisplatin dose escalation	50
Lin et al. *(77)*	Cisplatin, mitomycin C, 5-FU, and leucovorin	28
Jang et al. *(78)*	5-FU and cisplatin	29
Carr *(164)*	Gemcitabine	

5-FU, 5-fluorouracil; MMC, mitomycin C; SMANCS, styrene maleic acid conjugates of neocarzinostatin and mitomycin C; IFN, interferon.

*Similar survival.

Table 5
Some Randomized Clinical Trials Involving Transhepatic Artery Chemoembolization vs Other Chemotherapy for HCC

Author	*Year*	*Agent 1*	*Agent 2*	*Effects on Survival*
Kawai *(79)*	1992	Doxorubicin + embo	Embo	None
Kawai *(80)*	1997	Epirubicin + embo	Doxorubicin + embo	None
Watanabe *(81)*	1994	Epirubicin + embo	Doxorubicin + embo	None
Chang *(61)*	1994	Cisplatin + embo	Embo	None
Hatanaka *(82)*	1995	Cisplatin, doxorubicin + embo	Same + lipiodol	None
Uchino *(83)*	1993	Cisplatin, doxorubicin + oral FU	Same + tamoxifen	None
Madden *(84)*	1993	Cisplatin + ADMOS	5-epi-Doxorubicin	None
Chung *(85)*	2000	Cisplatin + 1FN	Cisplatin	None
Lin *(48)*	1988	Embo	Embo + IV FU	None
Yoshikawa *(86)*	1994	Epirubicin + lipiodol	Epirubicin	None
Kajanti *(87)*	1992	Epirubicin + FU	IV epirubicin + FU	None
Tzoracoleftherakis *(88)*	1999	Doxorubicin	IV doxorubicin	None
Bhattachariya *(89)*	1995	Epirubicin + lipiodol	^{131}I-Lipiodol	None

Table 6
Randomized Clinical Trials Involving Transhepatic Arterial Chemoembolization (TACE) Chemotherapy vs No Treatment Controls

Author	*Year*	*Agents*	*Effects on survival*
1. Pelletier *(55)*	1990	Doxorubicin + Gelfoam	None
2. Trinchet *(60)*	1995	Cisplatin + Gelfoam	None
3. Bruix *(90)*	1998	Coils and Gelfoam	None
4. Pelletier *(91)*	1998	Cisplatin + lipiodol	None
5. Lo *(92)*	2002	Cisplatin + lipiodol	Yes
6. Llovet *(93)*	2002	Doxorubicin + lipiodol	Yes
7. Reviews *(39,40, 94, 95)*			

2. SPECIAL CONSIDERATIONS FOR THE ONCOLOGIST

HCC arises on the basis of a diseased liver, which is more sensitive to toxic damage by chemotherapeutic agents than normal liver. In addition, cirrhosis causes portal hypertension, which poses additional hazards for the chemotherapist. These are described below.

2.1. Myelosuppression

Portal hypertension is associated with splenomegaly and associated leukopenia and thrombocytopenia. Unlike the myelosuppression that results from systemic chemotherapy and can be attributed to chemotherapy-mediated damage to the cells of the bone marrow, the leukopenia and thrombocytopenia consequent to splenomegaly are thought to be the result of sequestration of blood cells in the spleen, in the presence of a normal marrow. Although the starting values of WBC and platelets in the patient with cirrhosis are typically lower than are permitted in most cancer clinical chemotherapy trials, it is our experience that patients rarely come to any harm from chemotherapy with a starting WBC greater than 3,000/L, or platelet count greater than 40,000/mL. The recent introduction of granulocyte colony stimulating factors (CSFs), such as pegfilgrastrim (Neulasta) into clinical practice, means that the WBC can be restored to safe levels by the oncologist at will.

2.2. GI Bleeding

Portal hypertension is associated with esophageal and gastric variceal bleeding in addition to colonic bleeding. This is a hazard for the cancer chemotherapist to consider, since the consequence of the chemotherapy is often a decrease in platelet counts. Our experience is that preventive banding or sclerosing of varices does not appear to make any difference compared to treating the varices only after there is a bleed.

2.3. The Cirrhotic Liver Has Decreased Xenobiotic Metabolizing Capacity

The decreased metabolic capacity, particularly the ability to detoxify xenobiotics, results in increased half-life of many of the common chemotherapeutic agents. This can result in life-threatening prolongation in the myelosuppression. Careful dose adjustment to the individual tolerance of the patient needs to be taken into account by the experienced oncologist. Whereas most patients tolerate cisplatin, doxorubicin, or FUDR, prolonged and frightening thrombocytopenia can result from use of mitomycin C.

2.4. Decreased Liver Synthetic Activity Associated with Portal Hypertension

An increased prothrombin time from decreased synthetic capacity of the liver poses hazards for the vascular interventional radiologist. We typically treat patients with fresh frozen plasma or platelet transfusions for a platelet count below 50,000/L prior to femoral artery puncture, but any chemotherapy delivered with a baseline INR above 1.5 risks hepatocellular failure, in our experience, due to the failure of the diseased liver. A low serum albumin level, especially when associated with more than minimal ascites, is a poor prognostic sign, in our experience.

3. HEPATIC ARTERY CHEMOTHERAPY AND CHEMOEMBOLIZATION

Hepatic artery drug delivery as a semi-selective means for delivering high concentrations of drugs to the tumor:The hepatic artery delivery of drugs such as chemotherapeutic agents is done with two aims. First, since the HCC is supplied mainly by hepatic arterial blood in contrast to the portal delivery of blood to the underlying liver, this offers a semi-selective means for delivering drug to the tumor rather than to the underlying liver. In clinical practice, the resulting transient elevation of several of the liver function tests suggests that the underlying liver is not really spared. Second, delivery

of many drugs into the liver via the hepatic artery appears to result in much higher hepatic extraction of drug compared with systemic delivery. As a consequence, since most HCCs are vascular, quite high concentrations of drugs can be delivered to individual HCC tumor masses.

3.1. Commonly Used Drugs

Chemotherapeutic agents that have been commonly used in many centers include cisplatin, doxorubicin (Adriamycin), 5-FUdR, mitomycin C, in addition to the much lower experience with neocarzinostatin (SMANCS) and, gemcitabine (Gemzar) (Table 5). They have been used as single agents and in combinations, with (usually) or without some form of embolizing agent to produce chemoembolization or chemo-occlusion. However, there are few consistent data, nor is there agreement on the number of agents to be infused, with one or more than one agent, and which agent(s) are superior. Until this can be resolved, the evidence favors use of either cisplatin *(92)* or doxorubicin *(93)* (Table 6). The most commonly used agent in addition to chemotherapy is lipiodol (Ethiodol), which is an oily radio-opaque material that produces an emulsion with the injected drugs. This emulsion is believed to keep the drugs in longer contact with the tumor. There is also some evidence to suggest that higher response rates and prolonged survival are associated with use of higher doses of cisplatin compared to lower doses *(76*, *96)* (Table 7).

3.2. Hepatic Arterial Occlusion

Various agents have been introduced into the hepatic artery together with chemotherapy, in order to cause vascular slowing (occlusion) or embolization (TACE, transarterial catheter embolization). These include Gelfoam (a degradable gelatin sponge—our favorite), Ivalon (polyvinyl alcohol which is irreversible and more dangerous, in our experience), autologous blood clots, degradable starch microspheres (Spherex, a relatively safe and attractive product), microcapsules, collagen (Angiostat), and steel coils. Recently, particles of defined size ranges have been introduced, such as Embogold compressible microspheres (Biospheres) with particle sizes of 40–120, 100–300, and 300–500 μm. A study done in 47 patients showed higher responses, measured by the decrease in tumor size and vascularity, for the 100–300 μm particles compared to the other two particle sizes *(97)*. Our main experience has been with Gelfoam, Spherex starch spheres, and biospheres, since the first two are all degradable and they all appear to be minimally hepatotoxic and cause only transient vascular occlusion, allowing further chemotherapy sessions after several weeks. Lipiodol (Ethiodol) has been widely used, particularly in Europe and Japan. We have not noticed any particular added

Table 7
Effects of Hepatic Arterial Cisplatin Dose Intensity *(76, 96)*

Patients treated: 57

Cisplatin alone $n = 26$
Cisplatin + Gelfoam = 31

A. Responses (PR): Cisplatin alone 11/26 (42%)
Cisplatin + Gelfoam 18/31 (58%)

B. Effects of **response** on median **survival** (month) ± SE:

Responders	*Cisplatin alone*	*Cisplatin + Gelfoam*
	29.0 ± 3.5	25.5 ± 1.7
Non-responders	11.1 ± 1.5	15.6 ± 3.1
	$p < 0.0001$	$p < 0.003$

C. Effect of treatment type on median survival (month) ± SE:

Cisplatin alone	Cisplatin + Gelfoam
19.53 ± 6.3	30.73 ± 0
$p < 0.137$	

D. Effect of dose density on median survival (month) ± SE:

	Cisplatin alone	Cisplatin +Gelfoam
Dose≤125 mg/m^2/month	**9.9 ± 1.66**	**16.4 ± 2.8**
Dose≥125 mg/m^2/month	**19.5 ± 7.2**	**30.7 ± 0**
	$p < 0.07$	$p < 0.69$

effect of lipiodol to chemotherapy in terms of tumor response *(98)*. In addition, it often obscures the subsequent interpretation of CAT scans.We have therefore abandoned its use. A recent meta-analysis confirmed the lack of evidence for the use of lipiodol in TACE *(95)*. There was also a suggestion in the meta-analysis that polyvinyl alcohol particles may be better than the other agents used in TACE. But the analysis did not show any difference between the various chemotherapy agents. The hepatic artery approach is

based on two considerations. First, since the hepatic artery supplies more than 90% of oxygenated blood to the HCC, but the portal vein does similar for the underlying liver, this permits a selective drug delivery. Second, as the hepatic arterial flow rate is reduced by use of an embolizing agent, enhanced hepatic uptake has been shown *(166)* for many cancer chemotherapy drugs, especially FUDR, doxorubicin, and cisplatin, for which 10-fold to 100-fold increases in regional drug delivery have been shown, as arterial flow decreases.

3.3. Protocol for Chemo-occlusion Therapy of HCC

Our largest experience has been with cisplatin. This is based on the fact that it has moderate tumor shrinking ability and has minimal myelosuppressive activity compared with most other agents. This is a useful property in the setting of portal hypertension. It is also relatively well tolerated by the cirrhotic liver. It is usually given at a starting dose of 125 mg per meter squared (125 mg/m^2) of body surface area (BSA). This dose is essentially tolerated by everyone with a bilirubin of less than 1.5 mg/dL, a normal INR, and without gross ascites. Patients who tolerate this well, without change in their blood count or increase in their liver functions, typically have the dose increased after two or three cycles to 150 mg/m^2 and then to 175 mg/m^2. The cisplatin is given in 100 mL of normal saline and infused into the hepatic artery over 30 min, together with dexamethasone 20 mg (to limit hepatic inflammation), morphine sulfate 5 mg (for pain), as well as intravenous antibiotics (Ancef or Vancomycin) given prior to TACE. A pressure pump is used to deliver the drug. About 250 mL of 3% saline is given intravenously at the same time. In addition, the patients are aggressively given intravenous hydration. This is done using D5½ normal saline or just ½ normal saline with 20 mEq KCl/L at 250 mL/h for a minimum of 3 h. Once the patient is in the vascular procedure room, the fluid rate is increased to 2 L over 2 h immediately prior to the cisplatin infusion, together with immediate intravenous infusion of the diuretics 12.5 g of mannitol and 40 mg of furosemide during the cisplatin infusion. This diuretic regimen is designed to prevent cisplatin from being retained in the kidney and causing nephrotoxicity. Aggressive triple anti-emetics consisting of a combination of Reglan, Benadryl (or Kytril) or Anzamet, and Dexamethasone are all given repetitively for the next 24 h. Prior to cisplatin, we give a single intravenous dose of Kytril 1 mg (Granisetron) or Zofran 32 mg (Ondansetrone), together with dexamethasone (Decadron) 4 mg. After cisplatin, we give intravenous Reglan 2 mg/kg (Metoclopramide), Benadryl 25 mg, and Decadron 4 mg every 3 h for the next 12 h. Zofran is continued at 10 mg IV every 8 h, or Anzamet or Kytril. In addition, we give an intravenous bolus of sodium thiosulfate 9 g/m^2 immediately before the chemotherapy and a 6 h intravenous

infusion of 1.5 g/m^2/h afterward. This has resulted in essential disappearance of cisplatin-mediated ototoxicity and neurotoxicity. Intravenous hydration at 150 mL/h is continued post-chemotherapy until the patient is discharged from hospital. Patients are typically hospitalized overnight and discharged the following morning. However, whether they need to be kept as an inpatient overnight is not really clear. Most patients require some form of bolus intravenous morphine sulfate, typically 2 mg or 5 mg injections, every 3–4 h for two or three administrations after the vascular occlusion. The pain of the post-embolization syndrome is likely due in part to arterial spasm. Lab work is re-checked the morning following treatment for electrolyte imbalances or potassium or magnesium losses that need to be replaced, as needed.

Gelfoam sponge particles (not powder), which are made by cutting up Gelfoam sponge sheets with scissors and then autoclaved, are typically injected hepatic-arterially at the beginning of the administration of chemotherapy, half way through and again at the end of the cisplatin administration. The idea is to cause vascular slowing but never complete occlusion. We thus do not actually perform complete embolization. This has resulted in a much greater safety margin for our protocol. The arterial flow is monitored during the chemotherapy by regular bolus injections of angiographic dye to check the vascular flow. Gelfoam powder is thought to be too toxic and is not used in our institution. Similarly, Ivalon is not given because of its hepatotoxicity and irreversibility, limiting the ability to give future doses of chemotherapy. Details of the angiography are presented in Chapter 21.

The chemotherapy (TACE) is typically repeated every 8–12 weeks, depending upon the hepatic tolerance, the tumor response and recovery of the WBC, platelets, liver transaminases, or bilirubin, and on the time period for clinical patient recovery. The main toxicity appears to be tiredness and loss of appetite for 7–10 days post-treatment. We have found with this regimen of intravenous triple antibiotic and intra-arterial morphine sulfate that nausea and vomiting are minimal and hepatic pain is also limited. The patients thus do not typically fear their repeated treatments.

4. SAFETY CONSIDERATIONS OF HEPATIC ARTERY CHEMO-OCCLUSION

4.1. Unilobar Treatments Are Given

It is possible to safely give chemotherapy to the whole liver through the proper hepatic artery to an entirely normal liver with metastatic cancer. It is also possible to do this with multifocal bilobar HCCs with completely normal liver function and no ascites and in the complete absence of portal vein thrombosis, hepatitis, or cirrhosis. However, our experience is that the chemo-occlusion is much safer when only one lobe of the liver is given

TACE treatment at any one treatment session. This is now our standard operating procedure. The lobe of the liver with the maximum amount of tumor is normally selected for initial treatment and several treatments are given to this lobe until tumor control is achieved. Then, the other liver lobe is treated on subsequent treatment sessions.

4.2. Vascular Slowing Is Performed Without Complete Occlusion

Chemotherapy is given with regular pulses of embolizing materials to achieve vascular slowing, but complete occlusion of the arterial blood flow is avoided to minimize subsequent hepatotoxicity.

4.3. Drug Doses Are Tailored to Each Individual

Almost all patients with a bilirubin of less than 1.5 mg/dL tolerate cisplatin 125 mg/m^2. Doses on subsequent treatments can be escalated (Table 7) through 150 mg/m^2, to 175 mg/m^2, although few patients can tolerate the last. A completely normal blood count and no change in liver function tests are used as the bases for increasing the dose of cisplatin by one dose level on a subsequent treatment. By contrast, prolongation of a prothrombin time or elevation of the bilirubin to above-normal levels is normally used to decrease the cisplatin to 100 mg/m^2 on a subsequent treatment or down one dose level if a higher dose than the starting dose level has been used. A nadir WBC above 2,000 $\times$ 10^3/L or nadir platelet count above 40,000 $\times$ 10^9/L rarely requires a decrease in the dose of cisplatin on subsequent treatments. The timing of repeated treatments is somewhat arbitrary. A newly diagnosed patient is typically put on a schedule of repeat treatments every 6 or 8 weeks for the first two or three treatments, until some form of tumor response can be seen. After this point, the time between treatments is rapidly increased up to a maximum of 12 weeks, in order to decrease the risk of liver damage by chemotherapy in the presence of cirrhosis. We think that extending the intertreatment intervals beyond 12 weeks is associated with increasing likelihood of tumor growth. However, it is our experience that tumors that decrease by more than 50% of their size can stabilize without repeat treatments for many months, without re-growth.

5. RESULTS OF HEPATIC ARTERY CHEMOTHERAPY AND CHEMOEMBOLIZATION

We have evaluated the results of treating a large number of patients with cisplatin-based chemoembolization (TACE) and have evaluated them based on prolonged survival greater than 24 months, poor survival less than 4

months, or intermediate between these two (Tables 8, 9, and 10). We found that cirrhosis alone was not a good predictor of poor survival, as plenty of patients with cirrhosis were also in the best survival category. However, poor liver function, as judged by an elevated bilirubin, low albumin, or prolonged prothrombin time (INR) were all strongly associated with the poor survival category (Table 8). The main tumor characteristics that appeared to be important in HCC patient survival after TACE were portal vein invasion and very high alpha-fetoprotein (Table 9). Tumor size or numbers of tumors did not appear to be important in our series. By contrast, any form of partial response to chemotherapy, as judged by tumor shrinkage or decreased tumor vascularity on a triple-phase helical CT scan was strongly associated with the prolonged survival group (Table 9). Examples of this are shown in the CT scans and angiograms in Figs. 1, 2, 3, 4, and 5. It appears that there are two types of HCC response to chemotherapy. These are formal tumor shrinkage (WHO and RECIST criteria) as noted with other types of cancer (Figs. 3 and 4), as well as a decrease in tumor vascularity *(100, 101)* (Fig. 2).

Table 8
Cisplatin Hepatic Artery Chemoembolization: Prognostic Factors for Survival ($n = 155$)

	Patient characteristics (% pts) *Patient survival*		
	>24 months n = *49*	*4–24 months* n = *26*	*<4 months* n = *26*
Liver disease			
Cirrhosis	73	84	88
HBV	28	29	31
HCV	30	36	35
Alcohol	12	15	19
Labs			
Bilirubin < 1.6 mg/dL	96	71	42
Albumin > 3.4 g/dL	76	47	35
No ascites	92	90	38
INR < 1.2	80	60	31
Platelets > 150×10^9/L	71	55	27
Portal HT (CT)	35	45	85

Table 9
Cisplatin Hepatic Artery Chemoembolization: Prognostic Factors for Survival (*n*=155)

	Tumor characteristics (% pts) Patient survival		
	>24 months n = *49*	*6–24 months* n = *80*	*<6 months* n = *26*
Tumors:			
Unilobar tumors	29	15	8
Bilobar tumors	71	85	92
> 3 tumors	78	83	85
PV invasion	41	56	73
Vascular tumors	90	80	42
Any tumor >5 cm	76	83	85
Metastases (except LNs)	6	17	15
AFP > 100 Kng/mL	12	30	46
Response to chemotherapy:			
Chemoresponses (PR)	84	69	8
Tumor stability	16	25	4

Since response to chemotherapy appeared to play such an important part in enhanced survival in our large TACE patient experience, we retrospectively examined those patient or tumor characteristics that correlated with response to chemotherapy (Table 10). We found that the presence of cirrhosis was much higher in those patients who did not respond to any chemotherapy (79%), although plenty of patients who did respond to chemotherapy also had some degree of cirrhosis (64%). An important consideration was tumor vasculature, since only 5% of patients with tumors that were hypovascular on CT scan, but 85% of patients whose tumors were hypervascular on CT scan had responses to treatment, as judged by tumor shrinkage (Table 10). Portal vein thrombosis was also important, since 86% of the patients whose tumors progressed on TACE had main portal vein thrombus, compared with only 48% in the response category. As in survival, tumor numbers or maximum tumor size appeared to have no correlation with response or failure to respond to TACE (Table 10). The new era of kinase inhibitors and anti-angiogenic agents (Chapter 22) is forcing a re-evaluation of the significance of a decrease in tumor size (response by CT or MRI scan). This is both because responses in HCC correlate poorly with survival and because

Table 10
Cisplatin Hepatic Artery Chemoembolization: Factors Associated with Tumor Responses (*n*=155)

	PR	*Stable*	*Progress*
	n = 98 (63%)	*n = 29 (19%)*	*n = 28 (18%)*
Survival			
<6 months	2 (2.0%)	1 (3%)	23 (82%)
6–24 months	55 (56%)	20 (69%)	5 (18%)
>24 months	41 (42%)	8 (28%)	0
Cirrhosis			
No	34 (35%)	10 (34%)	6 (21%)
Yes	64 (65%)	19 (66%)	22 (79%)
Tumor vasculature			
−	5 (5%)	1 (3%)	14 (50%)
+/−	10 (10%)	5 (17%)	2 (7%)
++	83 (85%)	23 (79%)	12 (43%)
PV thrombus			
−	51 (52%)	17 (58%)	4 (14%)
+	47 (48%)	12 (41%)	24 (86%)
Number			No correlation
Maximum size			No correlation

the newer agents such as sorafenib enhance survival with minimal associated scan tumor responses *(162)*. Effort is now ongoing to develop semiquantitative algorithms for clinical measurement of changes in HCC vascularity (tumor blood flow), using dynamic contrast-enhanced MRI and dye-enhanced ultrasonography.

5.1. TACE Using Drug Eluting Beads

Drug eluting beads can deliver the chemotherapeutic agent gradually over a period of time. This has the potential to achieve better tumor response rates and decrease in vascularity. There have been studies done only with doxorubicin containing drug eluting beads so far. The results seem to be promising with response rates anywhere from 50 to 81%, similar to

TACE, and with a good safety profile *(102–104)*. No convincing survival data are yet available. Large randomized control trials in the future will be needed to give us definitive answers regarding the efficacy of these agents.

6. SYSTEMIC CHEMOTHERAPY

A huge number of randomized and non-randomized studies have been performed with various single agents and some combinations of chemotherapeutic agents (Table 3). In Table 3 there are also several reviews. The bottom line is that the typical response rates appear to be no greater than 30% of patients nor is there a survival benefit for any single agent thus far tested. Similarly, claims of enhanced responses up to 20% for some combinations such as PIAF *(107)* are associated with enhanced toxicity but it is not clear whether there is a survival benefit there either. For this reason, much of the recent literature has focused on regional chemotherapy to try and enhance tumor exposure to the cytocidal effects of higher doses of chemotherapy. The use of tyrosine kinase inhibitors like sorafenib, sunitinib, and erlotinib in advanced HCC are discussed in Chapter 22. Despite the promising data with these newer classes of agents, systemic chemotherapy may still have a role in combination with these newer agents or in treatment of patients whose tumors progress on tyrosine kinase inhibitors. Renewed interest in the systemic therapies for HCC has accompanied the recent publication of a 10-week survival advantage for oral sorafenib therapy in a large randomized trial *(162)* and even longer survival in a newly published phase II trial using a combination of bevacizumab plus erlotinib *(163)*.

7. OTHER SYSTEMIC THERAPIES

A variety of hormonal therapies have been assessed for their usefulness in shrinking HCCs or enhancing of the survival in HCC patients. This has been based on the known gender bias, in which HCC has been found to be a predominantly male disease and in which antigen receptors have been found in many HCC tumors. As a consequence, both tamoxifen and LHRH antagonists have been evaluated, as well as megesterol (Megace) for their tumor shrinking abilities (Table 11). Despite initial reports of responses to tamoxifen, subsequent controlled randomized trials have essentially shown no survival benefit for tamoxifen, LHRH antagonists such as leuprolide, flutamide, or megesterol. A similar large number of studies have investigated the effects of interferons because they have an anti-angiogenic action and an anti-hepatitis activity. Although there are conflicting reports of benefit or no benefit to tumor shrinkage or survival, the consensus is that there is

Table 11
Various Recent Medical Treatments Evaluated for Unresectable HCC

A. Systemic
Tamoxifen *(113–118)*
LHRH agonists *(119, 120)*
Interferon *(121–124)*
Sandostatin *(125–131)*
Megestrol *(132, 133)*
Vitamin K *(108, 134–136)*
Thalidomide *(137–143)*
EGFR antibody *(144)*
Arsenic trioxide *(145)*
IL-2 *(68)*
Anti-angiogenesis strategies *(146)*
Immunotherapy *(147)*
B. Hepatic arterial
^{131}I-Lipiodol *(148–151)*
^{131}I-Ferritin *(152)*
^{90}Yttrium microspheres *(153–158)*

no survival benefit for the use of interferon at any dose level including huge doses of interferon that would not normally be tolerated by Western patients. Vitamin K or its analogs are a very attractive therapy, since a biochemical hallmark of HCC is a defect in vitamin K metabolism, resulting in elevated levels of immature prothrombin or des-gamma-carboxy prothrombin (DCP or PIVKA-2), which is one of the more useful HCC serum tumor markers *(99, 108, 109)*. Although vitamins K1 and K2 appear to be almost nontoxic in adult humans, they have fairly weak antitumor activity, as judged by tumor responses, even given at supra-therapeutic doses. However, two recent randomized trials from Japan show that oral K vitamins can decrease post-resection recurrences, as well as decrease the incidence of HCC in HCV carriers *(110–112)*.

The concept, however, is attractive and it may only be a matter of time before more potent K vitamin analogs are introduced into clinical testing for the treatment of HCC. Cetuximab which is an epidermal growth factor antibody did not show any single-agent activity in advanced HCC. Minimal to no activity was found in studies involving single-agent thalidomide, octreotide, or arsenic trioxide (Table 11).

Although HCC is thought to be, in general, a radio-resistant tumor, there is some evidence of anti-tumor activity with radioactively administered

agents delivered into the hepatic artery including ^{131}I-lipiodol, ^{188}Re-lipiodol, ^{166}Ho, and ^{32}P (Chapter 21). These agents have only mild activity so far. ^{90}Yttrium glass spheres, either imbedded in a resin or in glass beads (TheraSphere®), have been used in the treatment of HCC. The main attraction of the pure beta-emitting agent with a 1 cm maximum path length and 62 h half-life is that very high doses of radiation can be given to vascular tumors with minimal hepatotoxicities so far *(153)*. In addition, only very small numbers of treatment applications are required, the tolerance is high, and the side effects are low. Thus, patients appear to have promising quality of life during such treatment. Figure 6 shows a CT scan demonstrating a complete response with this therapy and Fig. 7 shows survival, arranged by CLIP score in a single institution trial. We have recently completed the analysis of 99 patients who received this treatment modality for their advanced HCC and the results were compared to a similar cohort of 691 patients receiving repetitive TACE *(166*, *167)*. The survival benefit with single-dose ^{90}Yttrium was equivalent to repetitive TACE and further ^{90}Yttrium had the added benefits of lower toxicity and single-dose administration. The survival data are shown in Fig. 8. A randomized comparison of ^{90}Yttrium (TheraSphere or SIR-Spheres) with intrahepatic chemotherapy will be needed to determine whether one treatment or the other is associated with prolonged survival and increased quality of life (Further reading *(168*, *169*, *170)*).

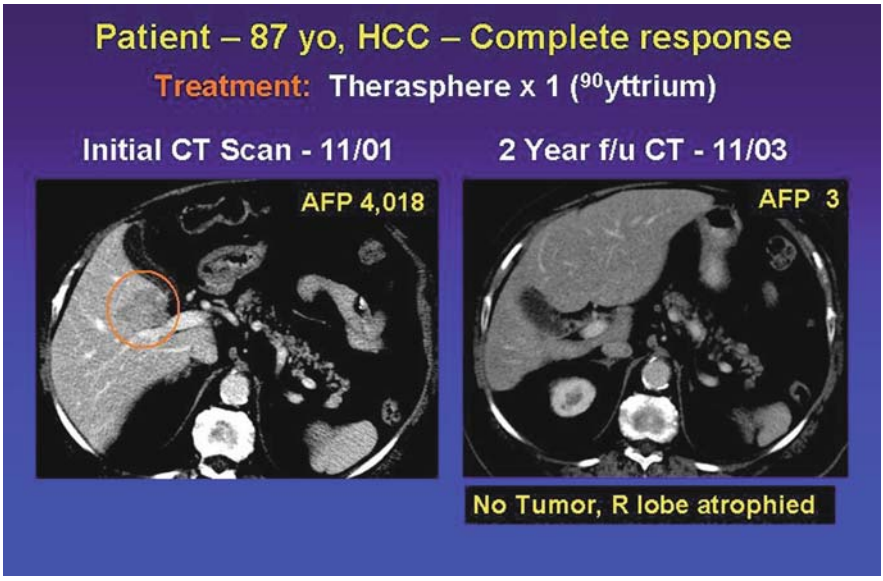

Fig. 6. CAT scan showing complete disappearance of HCC following Therasphere® therapy, accompanied by lobar atrophy.

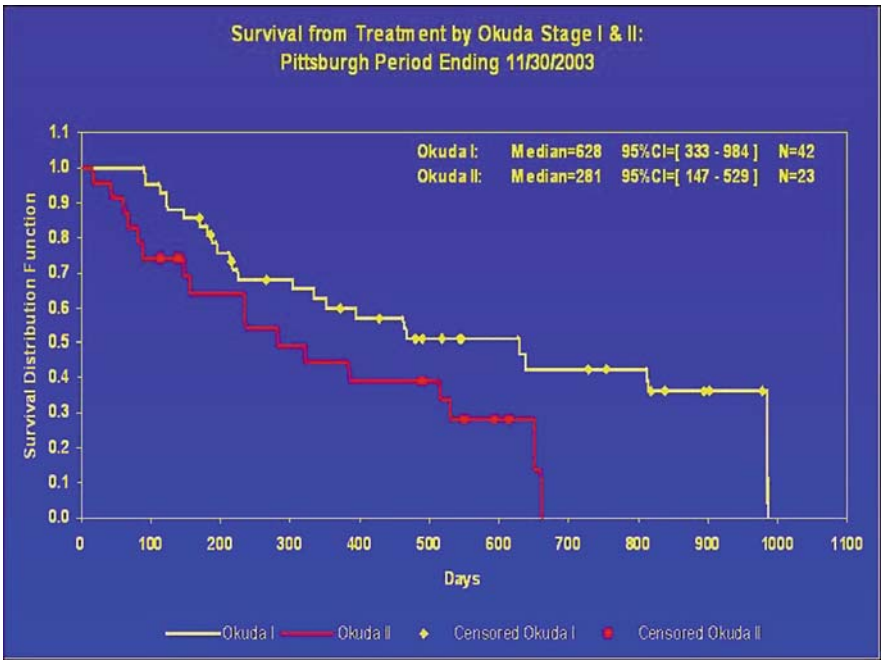

Fig. 7. Kaplan-Meier survival plot after HCC therapy with Therasphere®.

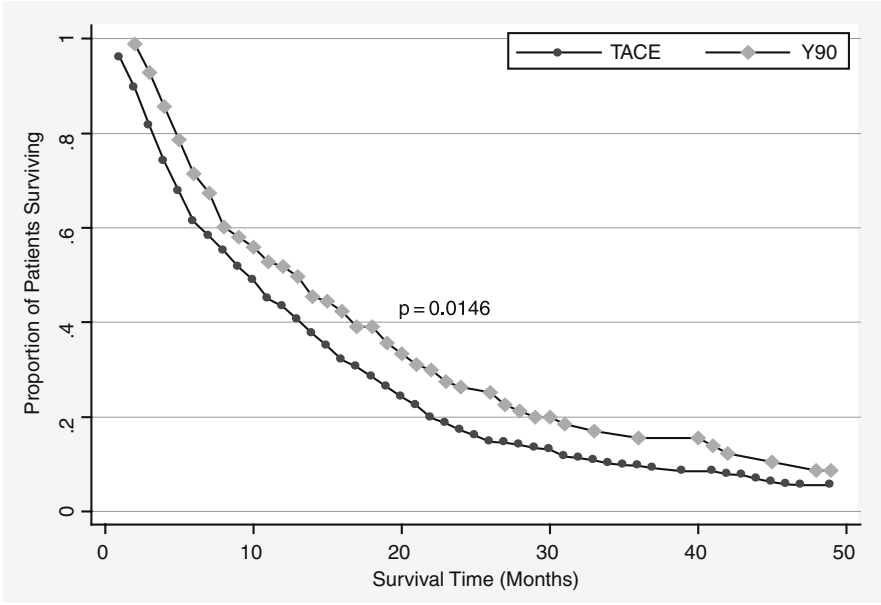

Fig. 8. Kaplan-Meier survival plot for 2 consecutive cohorts of HCC patients, treated with TACE ($n = 691$) or Therasphere(S) ($n = 99$).

8. WHAT IS NEEDED NEXT?

8.1. Improvements in Therapy of Unresectable HCC

The greatest need is the development of newer, more active drugs that have minimal hepatotoxicity. The anti-angiogenics and the cell cycle regulatory drugs appear to be attractive candidates.

8.2. Earlier Diagnosis

Given that survival by surgery is significantly enhanced for lower stage HCC compared to advanced stage HCC, screening programs resulting in earlier diagnosis with lower stage disease would be predicted to result in enhanced survival after treatment. Any screening program is predicated on knowledge of the etiological or predisposing factors for HCC development, as well as a long time interval between the action of such factors and the development of the tumor (as used in screening for carcinoma of the cervix uteri). Both of these criteria are satisfied for HCCs that develop on the basis of chronic HCV, chronic HBV, or cirrhosis from any cause, since one to two decades typically occur between infection and tumor development. Annual screening of patients by ultrasound or CT scan together with tumor markers (alpha-fetoprotein and DCP) might be expected to result in the diagnosis of tumors at an earlier stage of disease in these known to have predisposing risk factors, than most of the tumors currently presenting at our center.

8.3. Liver Transplantation Is Still Needed

Even if chemotherapy is completely successful in eradicating or inhibiting the growth of HCCs after diagnosis, more than 80% of the patients still have another chronic disease, namely cirrhosis. Since this probably plays a large part in the limited survival of patients with advanced stage HCC *(159)*, some form of liver replacement therapy is still needed for the treatment of HCC that is based on cirrhosis. Whether this is based on cadaveric donor liver transplantation, living-related donor liver transplantation, partial liver transplantation, hepatocyte transplantation, stem cell transplantation, or the ability to biologically reverse the fibrosis in a cirrhotic liver, these are all possibilities for the future total care of patients with HCC.

8.4. HCC Primary Prevention

The ideal long-term advance in HCC management would be cancer prevention entirely. This is feasible, given that we know the etiological cause in such a high percentage of these patients. Two obvious strategies are

immediately available and include vaccination and prevention of hepatitis or the treatment of chronic carriers of hepatitis, as well as refrigeration of stored food grains and peanuts (substrates for growth of fungi-producing carcinogenic mycotoxins, Chapter 2) in the Third World. In those Third World countries where HCC is most common, most of the population is agrarian and most food staples such as rice are stored in un-refrigerated village silos. After the monsoons, the high humidity encourages the growth of carcinogenic fungi, of which Aspergillus flavus-producing aflatoxins are only the best studied. The provision of refrigerated granaries for stored grains is expected to go a long way to reducing the conditions under which such carcinogen-producing organisms can flourish, and thus decrease the exposure and the risk of the population to hepatocarcinogens.

8.5. Causes of Death in HCC Patients

Why do patients with unresectable HCC die? It may seem obvious that they die because their growing tumors physically destroy the underlying liver. But most of these patients also have cirrhosis, which is a cause of death from liver failure even without presence of a tumor. Also, TACE is hepatotoxic, and several clinical trials have reported decreased survival in some patients after TACE therapy. In a recent analysis of our HCC patients' deaths, we gave ourselves the rule that if the CAT scan did not worsen or the alpha-fetoprotein did not increase in the 6 months prior to death, then the patient probably did not die only of cancer. On that basis, 42% of our patient deaths were not attributable to cancer growth *(159)*.

The field of primary prevention (HBV vaccination, Chapter 7), early detection (surveillance screening of people at risk cirrhosis), and the newer therapies (^{90}Yttrium, growth modulators, anti-angiogenics, Chapters 22 and 23) have brought renewed excitement to the field of HCC management, in which multiple ongoing clinical trials of newer therapies (including gene therapy) are already in progress.

8.6. Quantitation of Tumor Vascularity

The rapid incorporation into routine clinical practice of anti-angiogenic and kinase inhibitor agents that decrease tumor vascularity, often without much change in tumor size, is leading to radiological efforts to provide at least semi-quantitative new imaging measures or adaptations of CT, MRI, and ultrasound techniques that will hopefully become generally available in the next year or two. Multiple conference presentations have been made and standardization and validation of these newer clinical measurements are in progress.

8.7. Genomics and Proteomics of HCC

The rapidly expanding fields of both blood and tissue proteomics profiling and gene microarrays (Chapter 5) are permitting molecular classification of patients into differing prognostic groupings, who are otherwise clinically and pathologically similar. Especially with the use of cell cycle kinase inhibitors and anti-angiogenic agents, identification of the relevant activated pathway in tumor biopsies, or presence of elevated blood levels of growth factors or their receptors for growth or angiogenesis, is expected to permit more rational choice of therapeutic agent, and perhaps permit stratification of patients with differing gene expression profiles, to more properly analyze future clinical trials.

9. FUTURE DIRECTIONS

9.1. Needs for TACE Standardization

There are many published reports of TACE and its methods. No trial has ever shown the superiority of two, three, or four drugs over one. Nor is it clear which agent is best. Perhaps several drugs, such as cisplatin and doxorubicin, are equivalent. We also need to know whether two or three agents in combination are superior to one (in general in medical oncology, combining agents requires dose lowering of each component, to minimize additive toxicities). Furthermore, although most published series involve embolization, some series use either bland embolization without chemotherapy or chemotherapy infusion without embolization. In addition, several products have been used for the embolization or vascular occlusion process, including Gelfoam and biospheres—the most popular, but also blood clot and a range of particle materials and sizes. Although most published reports use ethiodol (lipiodol), this is based mainly on usage rather than evidence. One study even shows no added benefit for lipiodol *(98)*. In addition to agreement on the drug(s) to be used, there is little standardization of the doses, which range from the therapeutic to the homeopathic. Given that two published RCTs showed a survival advantage for single-agent cisplatin or doxorubicin when used for TACE *(92*, *93)*, it would seem that either should represent the current TACE standard for future trials.

9.2. Combinations of TACE with Kinase Inhibitors

There are currently two sets of standards for therapy of unresectable HCC. They are single-agent cisplatin or doxorubicin-TACE (above), which produce both tumor shrinkage (responses) and minor survival advantage on the one hand and oral kinase inhibitors, such as sorafenib, that produce minimal

tumor shrinkage, but up to median 2.5 months survival advantage, on the other hand. Results for just-published bevacizumab plus erlotinib look even more exciting *(160, 163)*.

Given the different modes of action between these classes of agent, it seems reasonable to evaluate the combination of these two classes of agents together, such as cisplatin-TACE plus sorafenib, doxorubicin-TACE plus sorafenib, intra-arterial ^{90}Yttrium plus sorafenib, or TACE plus bevacizumab and erlotinib. These combinations might result in the benefits of both tumor shrinkage and enhanced survival.

9.3. Adjuvant and Neo-adjuvant Therapies

The results of adjuvant chemotherapy trials for resection have been disappointing, apart from use of ^{131}I-lipiodol (see Chapter 17). In part, this may have been due to sub-therapeutic chemotherapy doses that were used in otherwise cancer-free patients. It may be that the new kinase and angiogenesis inhibitors will offer a better therapeutic margin and be useful in the adjuvant setting. As the criteria for liver transplantation get pushed toward offering this modality for multifocal tumors, there is a need for RCTs in the pre- or post-transplant setting. None have ever been published, even with chemotherapy. However, since only transplantation has the potential to simultaneously cure both the underlying liver disease and the tumor, there is a need for RCTs of chemotherapy, kinase inhibitors, or anti-angiogenics in the setting of liver transplantation. The need seems even greater for live-donor transplants, where the rules have been more generous and patients with more advanced tumors have been transplanted.

9.4. Newer Clinical Trials

About 340 clinical trials for HCC are listed on www.clinicaltrials.gov, of which 170 studies are currently recruiting HCC patients. They include the combination of TACE with sorafenib, ^{90}Yttrium microspheres, and new combinations of chemotherapies or chemotherapy plus biologics, such as capecitabine and oxaliplatinum, octreotide-LAR, TACE with lobaplatin and mitomycin C, everolimus, mapatumumab (TRAIL-1R Ab) plus sorafenib, cetuximab, bevacizumab, gemcitabine plus oxaliplatinum plus bevacizumab, TACE plus bevacizumab, ^{90}Yttrium (SIR-Spheres) plus sorafenib, gemcitabine, cisplatin plus sorafenib, some newer oral kinase inhibitors, new brachytherapies (^{32}P and ^{192}Ir), doxorubicin drug eluting beads, bevacizumab plus everolimus, brivanib, IGF1 receptor antibody, and several other newer agents in early phases of evaluation. A rich harvest of

new drugs and combinations of chemotherapies, biologics, or chemotherapies plus biologics is opening a field where few promising agents existed up to 5 years ago. This rapidly developing area will likely result in a different therapeutic landscape 5 years hence.

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21 Percutaneous Interventional Technique for Intra-arterial Chemoembolization

Nikhil B. Amesur, MD, and Albert B. Zajko, MD

CONTENTS

ABSTRACT

Transarterial chemoembolization techniques for hepatocellular carcinoma have been developed over the last couple of decades due to poor response from systemic chemotherapy in patients with liver tumors. In this chapter, the authors describe various aspects of intra-arterial chemoem-

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bolization starting with patient selection and preparation. Relevant normal and variant arterial anatomy is outlined. Catheterization techniques including advanced techniques are discussed. Embolization agents along with new horizons in treatment are highlighted. Complications from the procedure are also discussed.

Key Words: Chemoembolization; TACE, Loco-regional therapy; Intra-arterial therapy; Arterial Anatomy; Variant Arterial Anatomy; Complications

1. INTRODUCTION

Primary hepatocellular carcinoma (HCC) is one of the most fatal malignancies and has a current worldwide incidence of half a million cases. Furthermore, it is estimated that by 2019 in the United States alone there will be 34,000 cases of HCC per year *(1, 2, 3)*. Death due to liver metastases from other cancers is another leading cause of mortality from cancers worldwide. Whereas surgical resection and transplantation offer the chance for a cure in patients with HCC, most patients present to medical attention with advanced disease and are often not surgical candidates *(4)*. Response to systemic chemotherapeutic agents in these patients is poor and a multidisciplinary approach is crucial in the management of these patients. Loco-regional treatment options have gained popularity and offer the best chance of increased survival. In this chapter, we focus on percutaneous intra-arterial catheter-based treatment methods for the treatment of HCC and discuss some of the complications related to the procedure. These procedures have been described for over two decades in the literature *(5, 6, 7)*. Various institutions have evolved their own treatment protocols using various combinations of chemotherapeutic agents and embolization agents with varied success. The terminology alone in this field is confusing enough when one considers that there are at least five different types of basic procedures used in this category including transarterial chemoembolization, transarterial chemotherapy infusion, transarterial bland embolization, transarterial oily chemoembolization, and radioembolization. The literature is riddled with confusing terms such as TACE (transhepatic arterial chemoembolization) and HACE (hepatic arterial chemoembolization). Furthermore, segmental, lobar, and whole liver treatments are performed. To alleviate some of this confusion with regard to what treatment has been performed in evaluating clinical results, standardization of terminology and reporting criteria has been published and should be followed by the treating physicians *(8, 9)*. Following these reporting standards not only will allow better evaluation of results from different studies but also clearer line of communication among different investigator groups. Clini-

cal results from TACE have been previously published and are discussed in greater detail in other chapters in this book by the editor *(10, 11)*.

All these arterial treatment modalities exploit the fact that the liver has a unique dual blood supply. Whereas the majority of the blood flow to the liver is derived from the portal venous system, hepatic malignancies, in general,

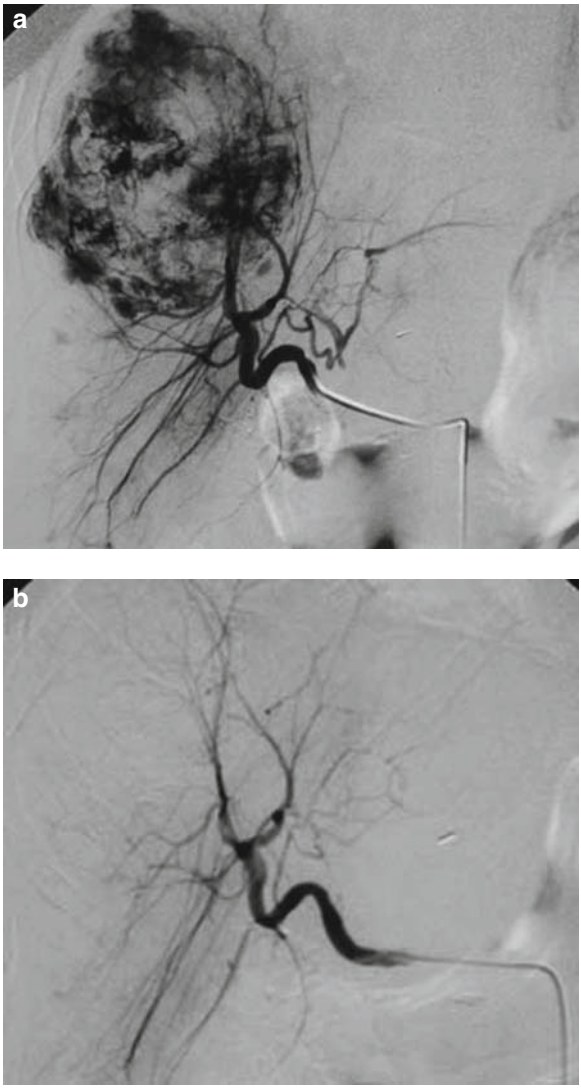

Fig. 1. (**a**) Right hepatic arteriogram shows the presence of a large hypervascular lesion in the right hepatic artery distribution. (**b**) Same patient as above after several cycles of chemotherapy infusion and gelfoam embolization no longer demonstrates presence of the hypervascular lesion.

tend to derive their blood supply solely from the hepatic artery (Fig. 1). This allows the interventionalist to selectively catheterize branches of the hepatic artery and deliver high doses of chemotherapy and other treatment agents directly to the malignant tissue. Since the agent is directly delivered to the liver, systemic side effects are often decreased.

2. PATIENT SELECTION AND PREPARATION

Patient selection for intra-arterial chemoembolization versus radiofrequency ablation versus other treatment modalities including surgical resection or transplantation is a complex process. At our institution as in other major institutions, all patients with liver tumors including HCC are presented at a weekly multidisciplinary tumor board. This conference is attended by the surgical oncologists, transplant surgeons, hepatologists, and radiologists. Furthermore, it provides a great learning opportunity for our residents and fellows across all specialties that are involved. All relevant radiological imaging and biopsy specimens are projected by the pathologist and radiologists. Relevant history including laboratory values, tumor staging, and the patients functional status is discussed. This allows the best treatment plan for each individual patient. In general, patients who are not candidates for surgical resection or radiofrequency ablation, those with advanced bilobar disease, those who have failed systemic chemotherapy agents, and patients with recurrent malignancy post-liver transplant are offered loco-regional chemoembolization at our institution. If all the appropriate criteria are met, the patient is scheduled to undergo the procedure in the interventional suite.

Patients are placed on a clear liquid diet from midnight prior to the procedure and are encouraged to hydrate themselves orally as much as possible. The patient reports to the outpatient unit early in the morning. Informed consent for the procedure and conscious sedation is obtained. Two large bore intravenous lines are usually placed. If the patient has a venous tunneled catheter or port, these can be used for one or both accesses. Intravenous fluid hydration is administered for 2–4 h at a rate of 250 cc/h if cardiac status will allow it.

A Foley catheter or a condom catheter is placed to allow adequate monitoring of urine output during and after the procedure. Mild sedative prior to Foley catheter placement is used. Intravenous antibiotics and anti-emetics are administered. Oral anxiolytics are given as needed prior to the patient being brought over to the angiography suite to reduce procedure-related anxiety.

Just prior to the procedure all cross-sectional imaging studies such as CT and MRI are reviewed by the treating physician. Visceral arterial anatomy is evaluated. In these patients we often just infuse the chemotherapeutic agent

only or embolize them to just slightly slow antegrade flow, in an attempt to minimize hepatic toxicity.

3. HEPATIC ARTERIAL ANATOMY ESSENTIAL TO TACE

Considerable variation exists in the arterial supply to the liver, and variant hepatic arterial anatomy may be seen in up to 42% of patients *(12, 13)*. The interventionalist should be intimately familiar with variant visceral arterial anatomy prior to undertaking these procedures so as to ensure that no vessels are left untreated and to avoid non-target exposure to these toxic substances.

Classically, the celiac axis gives rise to three vessels: the splenic, common hepatic (CHA), and left gastric arteries (LGA) (Fig. 2). The CHA gives rise to the gastroduodenal artery (GDA) and then becomes the proper hepatic artery (PHA). The PHA divides most commonly into the left (LHA) and right hepatic arteries (RHA). A single LHA may divide into a medial segment and a lateral segment or they may originate independently from the PHA or in some cases from the CHA. The cystic artery most commonly arises from the RHA. The caudate lobe of the liver is usually supplied by branches from the right hepatic artery. The GDA gives rise to the superior pancreaticoduodenal artery (sPDA). The inferior pancreaticoduodenal artery (iPDA) is usually a branch of the superior mesenteric artery (SMA). Both the sPDA and iPDA have anterior and posterior divisions, which join together to form the pancreaticoduodenal arcade (PDA). These collateral pathways between the celiac axis and SMA become especially important in cases of celiac artery or common hepatic artery occlusion, where alternate routes of selective hepatic artery catheterization may have to be exploited.

4. VARIANT ARTERIAL ANATOMY

As many as 20% of patients may have some vascular supply to the liver originating from the SMA. Variations include replaced right hepatic artery from the SMA (Fig. 3), accessory right hepatic artery (in which case there is a main right hepatic artery originating from the celiac trunk), or replaced common hepatic artery (Fig. 4). Patients may have the entire left hepatic artery or the left lateral segment hepatic artery originating from the left gastric artery (Fig. 5) or arising from the SMA (as part of a replaced common hepatic artery – 2.5%). The common hepatic or right hepatic artery may also rarely originate directly from the aorta. Arterial anatomy may also be confounded when the patient has undergone an orthotopic liver transplant and is being treated for tumor in the allograft. These patients may have an arterial

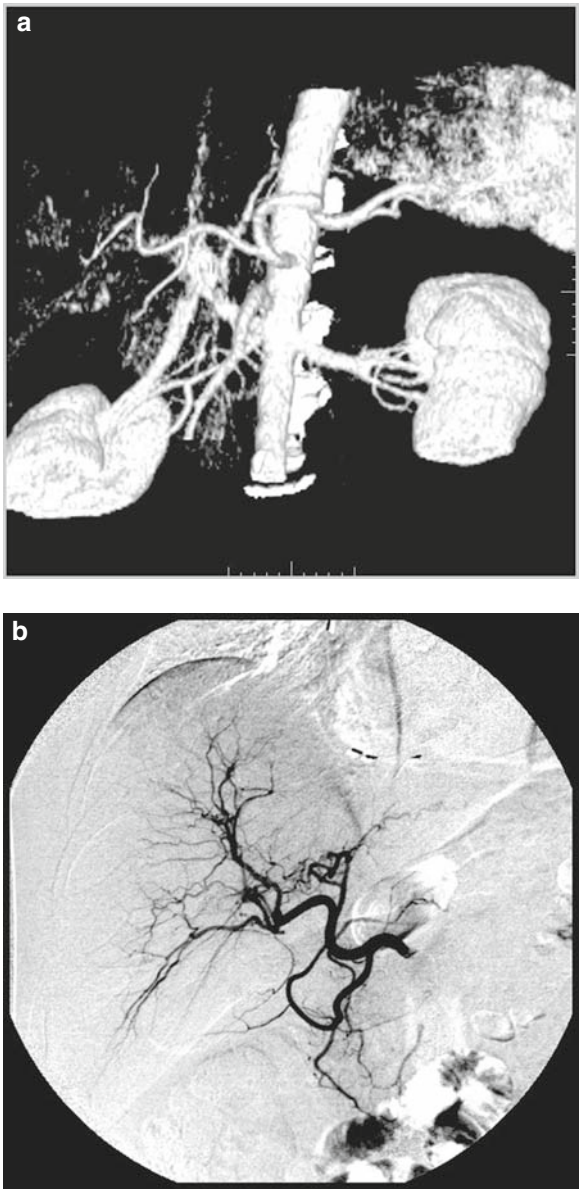

Fig. 2. (**a**) CT arteriogram demonstrating conventional hepatic artery anatomy. (**b**) Common hepatic arteriogram demonstrates similar appearance of the common hepatic artery.

graft directly arising from the infra-renal aorta. The operative note in these patients should be reviewed prior to treatment.

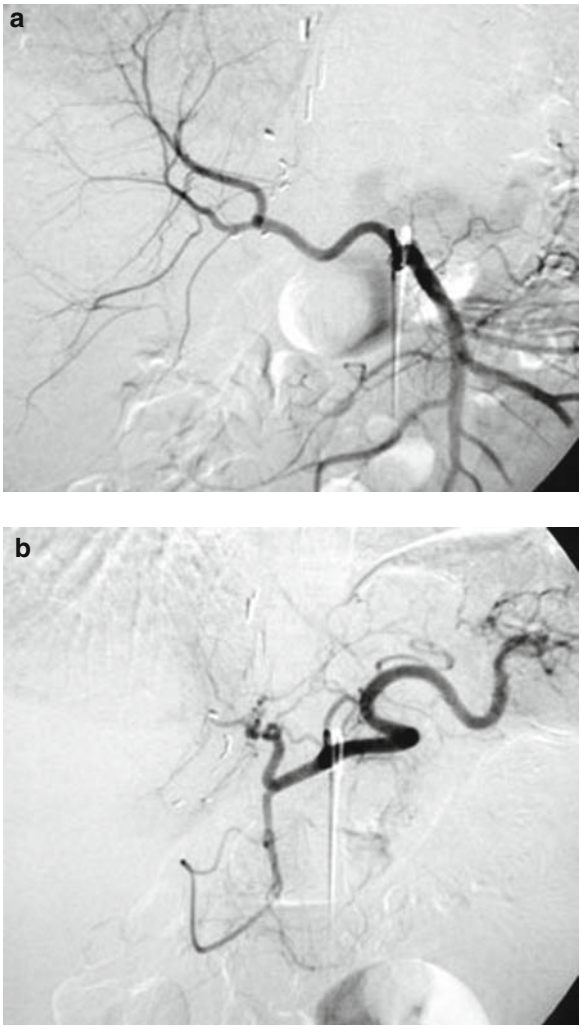

Fig. 3. (**a**) Replaced right hepatic artery seen originating from the SMA. (**b**) Same patient as above demonstrates only the LHA, GDA, and splenic artery from the celiac axis.

5. PROCEDURE

The patient is placed on cardiac monitors and administered moderate intravenous conscious sedation, usually a combination of an anxiolytic such as midazolam HCl, and pain medication such as fentanyl citrate. A small percentage of our patients are unable to undergo the procedure with conscious sedation, and deeper sedation or general anesthesia by the Anesthesiology department is utilized. The femoral artery is accessed using a

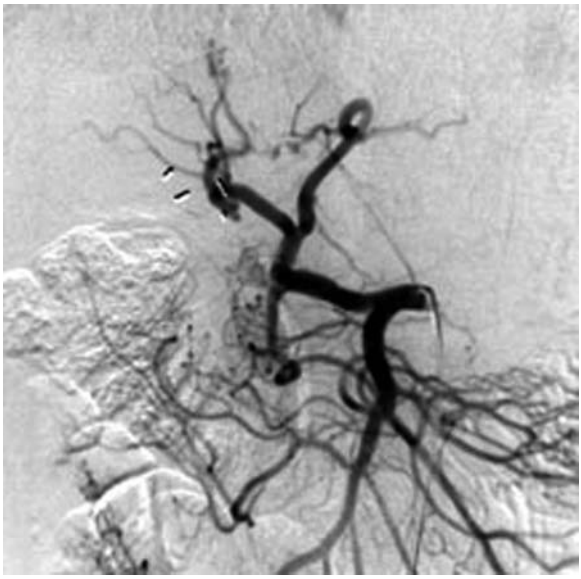

Fig. 4. Replaced common hepatic artery arising from the SMA.

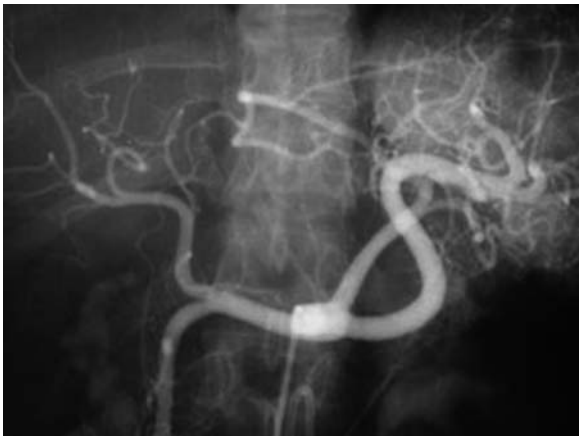

Fig. 5. Replaced *left* hepatic artery seen originating from the *left* gastric artery.

standard Seldinger technique or single wall technique often using a 21-gauge needle from a micropuncture set (Cook Inc, Bloomington, IN), especially if there is a coagulopathy or thrombocytopenia present. A sheath may be used depending on operator preference. A Cobra 2 or Sos catheter (Angiodynamics, Queensbury, NY) is used to catheterize the origin of the SMA. SMA arteriogram using either digital subtraction angiography or just a hand

injection of contrast under fluoroscopy is performed to exclude the presence of replaced hepatic vasculature off the SMA such as a replaced RHA or an accessory RHA or even a replaced CHA originating from the SMA. This needs to be assessed for, since missing such a replaced vessel can result in incomplete treatment of the tumor. In many cases, this information can be predetermined from the arterial phase of the CT. However, in all our patients we still pursue an SMA injection during the first treatment session and document this in our dictated reports obviating the need to recheck this on subsequent treatment sessions.

The celiac axis is then cannulated and a celiac arteriogram is performed to get a general overview of the celiac trunk and to look for a replaced left hepatic artery off the left gastric artery. In some patients the celiac axis can have an acute angle at its origin, such vessels often need to be accessed either using a reversed curve catheter such as a Sos catheter (Angiodynamics) or by creating a Waltman loop *(14)*. Briefly, the Waltman loop is created by advancing the C2 catheter into the opposite iliac artery and then a removable core straight wire is advanced to the end of the catheter. The core wire is pulled back making the end of the wire and catheter floppy. Using a series of twists and advances the catheter is advanced up into the aorta. This results in the C2 catheter bending back on itself in the aorta. Once this is achieved the catheter is pulled back down into the aorta and the celiac axis cannulated. The catheter can then be directed toward the vessel that needs to be entered.

A complete hepatic arteriogram is performed. If conventional hepatic arterial supply is present, this can be accomplished by catheter placement in the common hepatic artery. Tumor vascularity is assessed and compared to that seen on cross-sectional imaging studies. Once data is collected a decision is made in conjunction with the oncologist as to which vessels need to be treated first and if concomitant embolization agents are to be used. Diffuse bilobar lesions are treated at our institution with single lobar treatment by alternating the lobes. Single lesions that are demonstrated on arteriography to be fed by one vessel may be treated superselectively using microcatheters. Once this decision is made, the appropriate vessel is cannulated. If the vessel to be treated is large enough and is not convoluted, the 5 Fr C2 catheter is negotiated out into it usually with the aid of an angled glide wire (Boston Scientific, Natick, MA). If the vessel is of a smaller caliber or is tortuous, microcatheters are employed.

There are many microcatheter choices available to the interventionalist. We most often use the Renegade Hi-Flo (Boston Scientific) microcatheter. Microcatheters with angled tips are also now available. The Renegade Hi-Flo catheter has a large inner diameter and can accommodate 0.018 in. diameter. If the vessels are smaller, various catheters designed for coronary and neurointerventional procedures can be used. Numerous guidewire choices

are available. Most often with the Renegade Hi-Flo catheter we use the Glidewire Gold (Terumo Medical, Somerst, NJ) or Headliner wire (Terumo Medical). Each one of these different catheter and wire combinations have their own advantages and the choice often depends on vessel size, tortuosity, and operator preference.

Once final catheter position is confirmed by contrast administration we save an image on the screen so as to monitor for catheter migration during the procedure.

We then administer 5 mg of morphine sulfate and 20 mg of Decadron intra-arterially into the vessel being treated. The morphine is used by us to reduce the pain associated with embolization, and the Decadron is used to decrease the inflammatory response created by the chemotherapy in the liver parenchyma.

The chemotherapeutic agent which is usually premixed in our pharmacy arrives in a saline bag with a total volume of 150–200 cc. We then attach the chemotherapeutic agent via an infusion pump to the catheter through a three-way stopcock. The stopcock allows us to infuse contrast through the catheter if we suspect that the catheter has moved during the infusion of the agent. We tend to infuse the chemotherapeutic agent at a rate of 300–350 cc/h, allowing the entire agent to be infused over 30 min. Periodic fluoroscopy is performed every 5 min or so to ensure that the catheter does not migrate. This means that the patient must remain on the angiography table during the chemotherapy infusion. Once the agent is infused, saline is flushed through the line to ensure that the entire agent is flushed into the patient. The next step is embolization, for which a variety of agents are available as outlined in the next section. The embolization particles, be it gelfoam or defined size microparticles, are then infused into the vessel under fluoroscopy to occlude antegrade flow in most patients and slow down flow in patients with elevated bilirubin levels. If the bilirubin level is not elevated and the portal vein is patent, we generally completely occlude antegrade flow with the embolic agent. Once this is done, the catheter is removed, hemostasis obtained, and the patient is admitted for overnight observation and continued hydration. In general, we try not to use arterial closure devices due to the frequency of repunctures. However, many new closure devices allow repunctures and this is based on operator preference.

6. EMBOLIZATION AGENTS

Over the years there has been an evolution of embolization agents. Initially, the most commonly used embolization agent at our institution for these patients was gelfoam (Surgifoam, Ethicon, a Johnson & Johnson Company, Somerville, NJ). This was a temporary agent that causes cessation of

flow in the vessel and then over the next few weeks breaks down allowing restoration of flow to the area as long as the organ was not infarcted. The gelfoam had to be hand prepared since the commercial product came in a 2 cm by 6 cm gelfoam wafer. This was prepared by us as demonstrated in the accompanying figure. The gelfoam is pressed down and then cut into 1–2 mm longitudinal strips using a pair of scissors (Fig. 6). The strips are then cut at a 90° angle so as to form the 1–2 mm pledgets. A mixture of 50% contrast and saline is mixed in with the pledgets just prior to use and allowed to soak in it. The syringe is then attached to another syringe by means of a three-way stopcock. The gelfoam slurry is forced back and forth between the syringes resulting in further breakdown of the gelfoam. The stopcock can be partially turned off in order to decrease the size of the hole that the gelfoam is forced through. This results in gelfoam fragments of a smaller size. This is especially important when embolizing through microcatheters to prevent clogging the catheter. Unfortunately, one often ends up with particles of different non-reproducible sizes. In our experience there was no long-term permanent occlusion with gelfoam.

The last decade has seen the introduction of pre-defined size embolic particles that permanently occlude the vessel. One such particle that we evaluated was the Embogold Microspheres* (EMBS, Biosphere Medical, Rockland, MA). Using predetermined sized particles certainly was clinically appealing. However, their permanence was not. Furthermore, when we initially used gelfoam we used to pre-embolize the feeding vessel in order to slow the passage of the chemotherapeutic agent, this caused us some concern with the more permanent agents, since we were concerned that the chemotherapeutic agent may not get to the lesion. We changed our practice to administering the embolization agent at the end. With several different size particles available for clinical use, the next question was what the optimal size of the embolic agent should be. Our prior published experience has shown that we got the best results with the mid-sized 100–300 μm size particles *(15)*. Other similar particles that are commercially available within the United States included the Contour SE (Boston Scientific) and the Bead Block (Biocompatibles, UK).

The next step in evolution of predetermined size particles has been realized in the last few years with the introduction of the DC Bead (Biocompatibles, UK; Surrey, UK) embolic microsphere particles. These particles have a unique characteristic in that they are capable of being loaded with anthracycline-based compounds such as doxorubicin chemotherapeutic agents and then slowly releasing it over time *(16)*. Initial studies have demonstrated them to be safe and effective in treating patients *(17, 18)*. Randomized trials comparing them to conventional intra-arterial treatment regimens are currently pending.

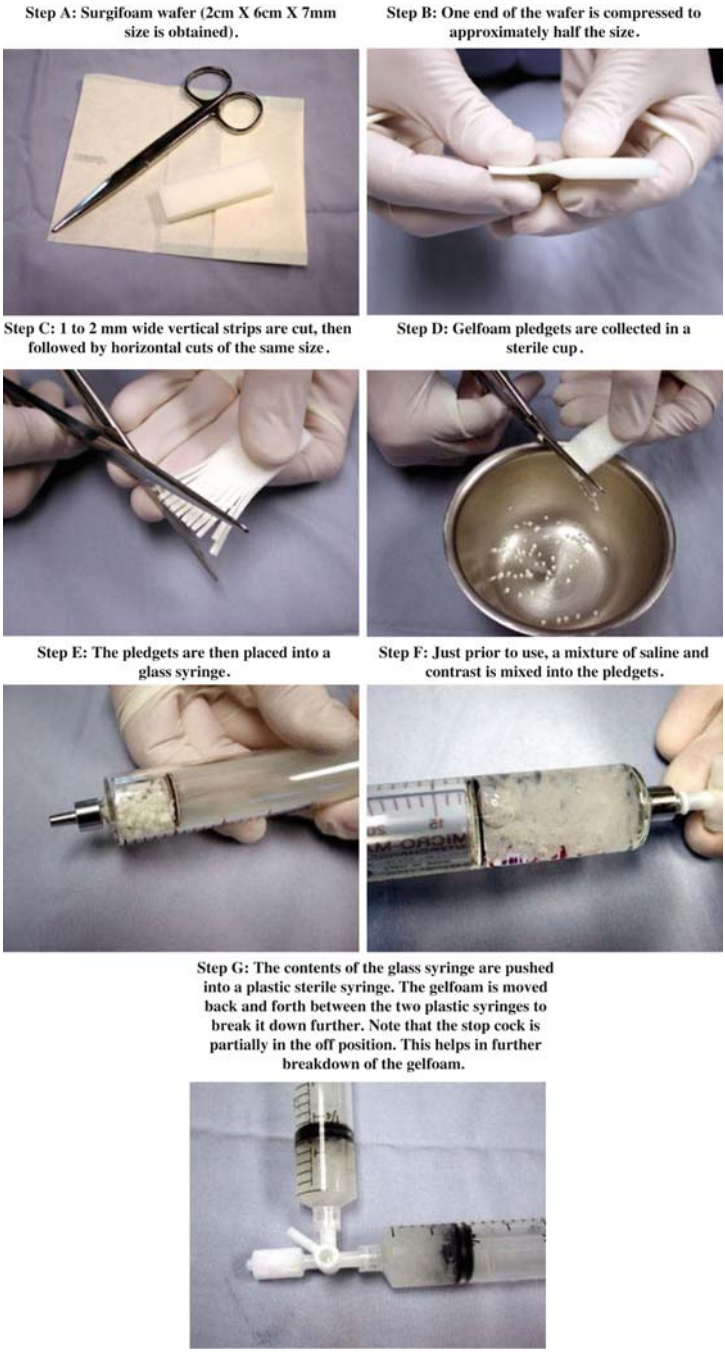

Fig. 6. Gelfoam preparation.

7. COMPLICATIONS FROM TACE

Various complications can arise as a consequence of TACE *(19)*. These can be broken down into direct procedural-related complications such as arterial dissection or occlusion, contrast reaction, puncture site hematomas, inadvertent chemoembolization of adjacent organs, and liver infarction. Late complications include liver failure, abscess formation *(20, 21)*, and chronic arterial occlusion.

Complications related to catheterization such as hematomas, arterial dissections, and occlusions are relatively small in the hands of experienced interventionalists occurring in less than 2% of patients. In the case of hepatic arterial occlusions, an experienced angiographer with knowledge of collateral pathways can take advantage of collateral pathways to still catheterize blood vessels feeding the tumor. Collaterals can arise from all adjacent vessels such as the phrenic arteries, branches of the internal mammary arteries, and branches from the pancreaticoduodenal arcade and have to be aggressively sought after.

Inadvertent chemoembolization of adjacent organs can easily occur unless meticulous attention is paid to the patients vascular anatomy. Patients have to be monitored closely for any clinical signs of non-target chemoembolization, such as ulcerations and bowel ischemia.

Abscess formation rates after TACE have a variable incidence with published data indicating a range of 0.2–4.5%. It is felt to result from ascending biliary infection post-TACE *(10, 11)*. A prior surgical biliary anastomosis may lead to increased risk of abscess formation. Prophylactic antibiotics especially to cover gastrointestinal flora prior to TACE are imperative to minimize this risk.

8. ADVANCED CATHETERIZATION TECHNIQUES AND ADJUVANT THERAPY

Complex anatomy can be encountered in patients. Also once a patient has undergone multiple TACE procedures, occlusions of the vessels can result. These may be related to the toxicity of the drugs used, repeated catheterizations, or tumor encasement. Once this occurs collateral vessels usually develop and angiographic assessment will often provide information on alternate arterial access. In many cases TACE is still possible through the use of collateral vessels *(22)*. If a celiac or CHA occlusion occurs, flow will often reverse in the GDA. This can be exploited by advancing a microcatheter up from the iPDA to the sPDA and up the GDA to catheterize the LHA or RHA (Fig. 7). Collaterals may also arise from the inferior phrenic artery or from the internal mammary artery (Fig. 8). In rare instances adjacent vessels may be recruited by the tumor and may need to be sought after

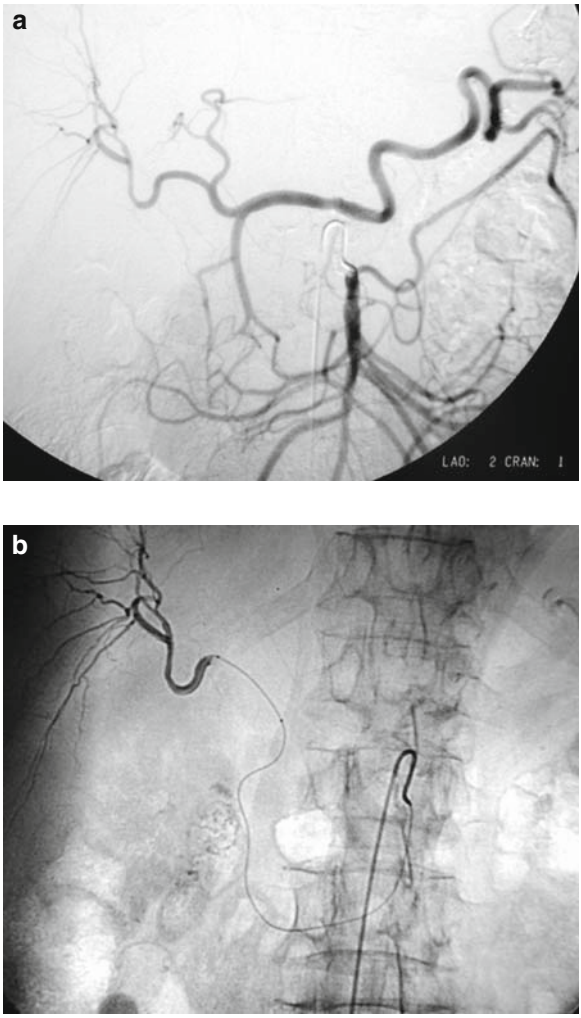

Fig. 7. (**a**) SMA arteriogram demonstrates complete filling of the celiac axis via the pancreaticoduodenal arcade, indicating complete occlusion of the celiac axis. (**b**) Tracker microcatheter has been advanced via the pancreaticoduodenal arcade all the way into the right hepatic artery for selective chemoembolization.

if treatment is to be continued (Fig. 9). These may need to be selectively assessed and catheterized.

In some patients stenoses of the intra-hepatic arterial branches or PHA may make selective catheterization impossible. The PHA bifurcation may arise very close to or at the level of the GDA. In such cases, the CHA may

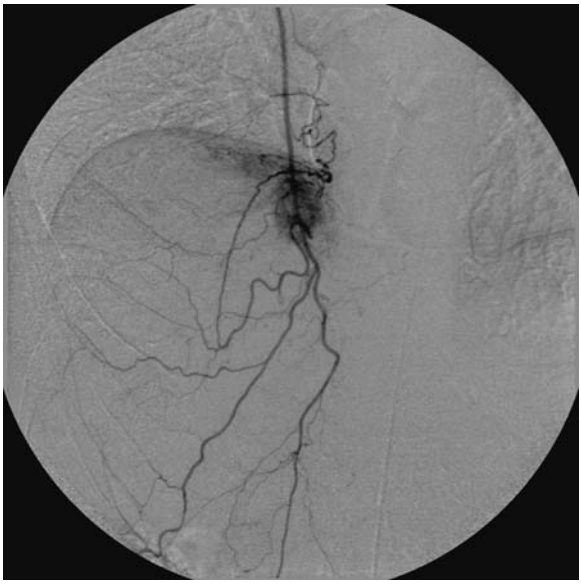

Fig. 8. Right internal mammary arteriogram through the right subclavian artery demonstrates branches supplying tumor in the superior aspect of the right lobe of the liver.

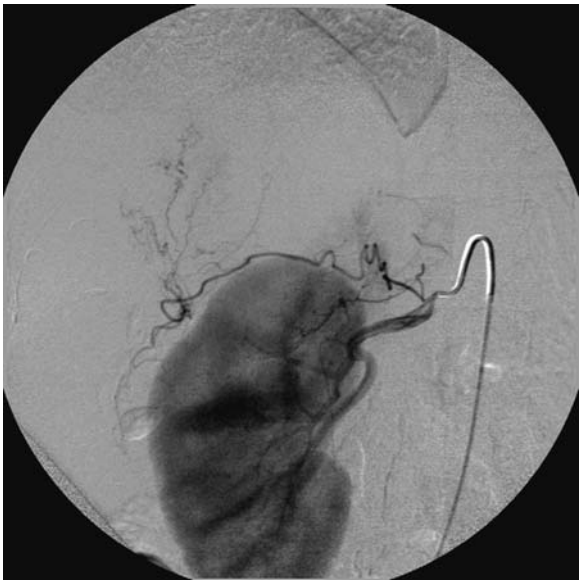

Fig. 9. Adrenal artery branch from the right renal artery supplying tumor blush within the liver.

need to be treated with TACE. Since chemotherapeutic agents are toxic to small bowel the GDA can be embolized using coils, effectively changing the CHA to the PHA, allowing bilobar treatment with a single catheterization.

The right or left hepatic artery may be occluded either due to repeated catheterizations or tumor ingrowth. Most of these patients will develop intrahepatic collaterals from the other side of the liver. In such cases, the opposite side treatment may be needed to allow chemotherapy cross flow from such collaterals. These patients have to be monitored closely since one would essentially be performing whole liver treatment.

9. RADIOEMBOLIZATION OF LIVER TUMORS

Catheter-directed delivery of a local burst of radiation offers an exciting treatment modality. ^{90}Yttrium glass spheres, either imbedded in a resin or in glass beads (TheraSphere; MDS Nordion, Ottawa, Ontario, Canada), has been used at our institution for a few years *(23)*. The use of TheraSphere and other similar beta-emitters may prove to be a more effective and tolerable treatment modality. When such beta-emitters are used care must be taken to limit its infusion only to the liver and care must be taken to ensure that there is no extra-hepatic delivery of the agent. Initially, we used to embolize various side branches such as the cystic artery prior to treating these patients but no longer do this as our experience with this modality has grown (Fig. 10). The details of this therapy are discussed in another chapter in this book.

10. CONCLUSION

Intra-arterial chemoembolization offers an effective method of delivering high doses of chemotherapeutic agent directly to the area of the liver affected by the tumor as well as embolizing the vessels in order to decrease tumor vascularity and increasing tumor necrosis (Fig. 11). Optimal treatment requires experienced angiographers with expertise in the use of microcatheterization techniques. A close working relationship needs to exist between the various disciplines that traditionally treat such patients at your institution. Multidisciplinary approach to each patient is important to see what each group can provide for every single patient. Furthermore, as the patient's tumor shrinks, re-presentation at these tumor boards is important to see if surgical resection or even transplantation criteria can be met essentially curing the patient from a life-threatening tumor.

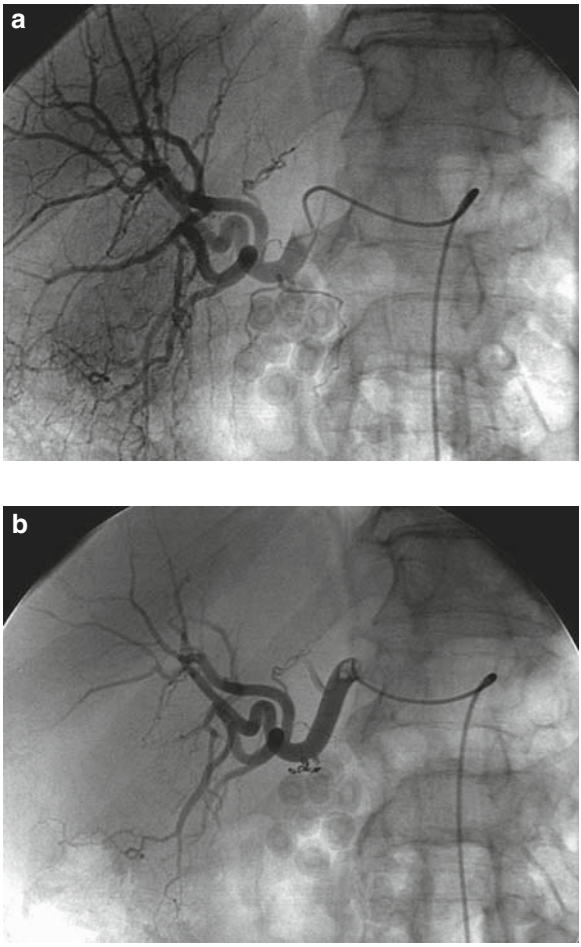

Fig. 10. (**a**) Selective right hepatic artery arteriogram showing filling of the cystic artery. Note the presence of gallstones in the gallbladder. (**b**) Selective right hepatic artery arteriogram after coil embolization of the cystic artery.

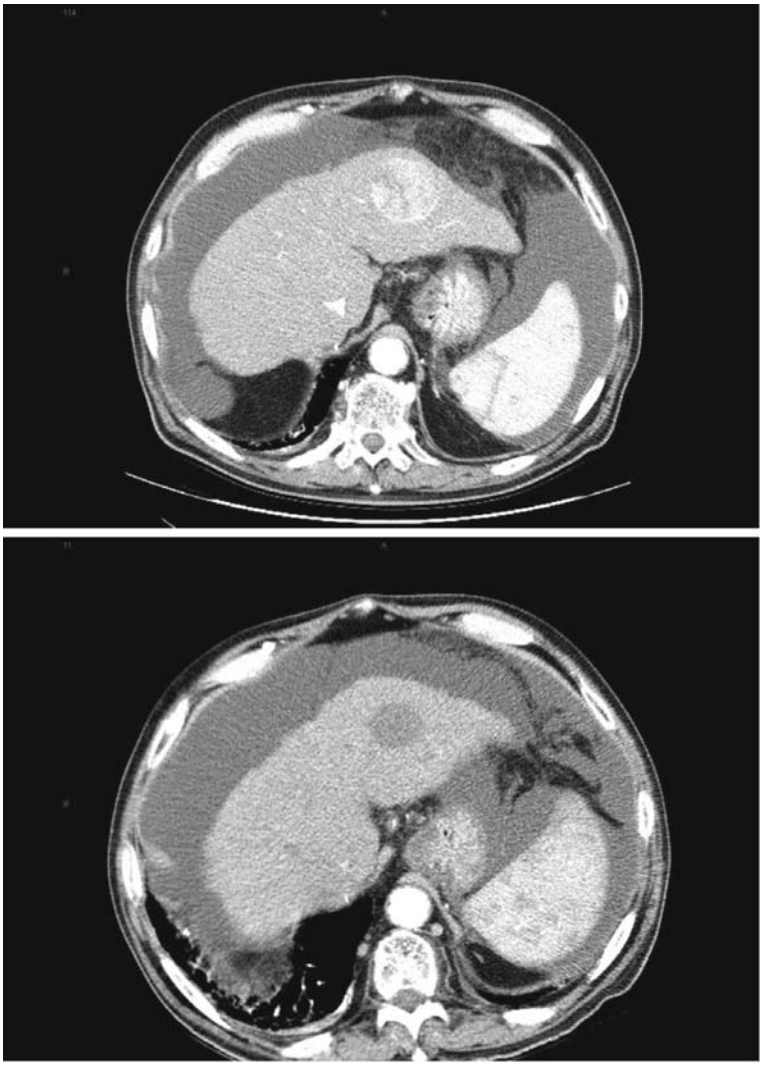

Fig. 11. CT scan before and after two cycles of intra-arterial chemoembolization demonstrating a striking decrease in tumor vascularity in left lobe HCC.

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22 Molecular Targeted Therapies for HCC

Brian I. Carr, MD, FRCP, PhD
and Susan Kralian PhD

CONTENTS

ABSTRACT

The years of fundamental cancer biology research is now starting to pay clinical dividends, with the identification of key enzymatic steps in the growth control and angiogenesis pathways, resulting in specific chemical inhibitors and antibodies to several of the involved kinases. This has led to a whole new family of novel, non-chemotherapy agents that are being tested alone or in combination with each other and with chemotherapy. Enhanced survival has been shown for at least one phase II and one phase III trial. These agents also have potential use in adjuvant therapy post-resection. Furthermore, they are changing our paradigms, as they seem to enhance survival without causing tumor shrinkage.

Key Words: Angiogenesis; cell cycle; oral new agents; combinations; sorafenib; erlotinib; bevacizumab

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Over the past 30 years, researchers have claimed victory in the war against cancer several times. Advances in molecular biology have led to an increased understanding of the discrete cellular pathways that promote or reduce cell division, cell survival, apoptosis, and angiogenesis. With the increased comprehension of the molecular etiology of cancer and these pathways, the era of rational therapy—the design of molecularly targeted agents that could modulate these cellular pathways (reactivate apoptosis and decrease cell growth, cell survival, and angiogenesis) to stabilize or halt the progress of cancer—began. Only in the past few years has this new knowledge and approach led to the production of pharmacologic agents that not only target a pathway but also produce clinical benefits.

Understanding molecular pathways can lead to the development of new drugs or improved drug regimens. Molecular pathways associated with hepatocarcinogenesis that modify apoptosis, cell division, cell survival, and angiogenesis include the rat sarcoma/rat sarcoma-activated factor/mitogen-activated protein kinase/extracellular regulated kinase (Ras/Raf/MAP/ERK) pathway, the phosphatidylinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway, Wnt/β-catenin, and the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (Fig. 1) *(1)*. These pathways are the targets of rational drug design, with the objective of modulating them to prevent progression or worsening of hepatocellular carcinoma (HCC).

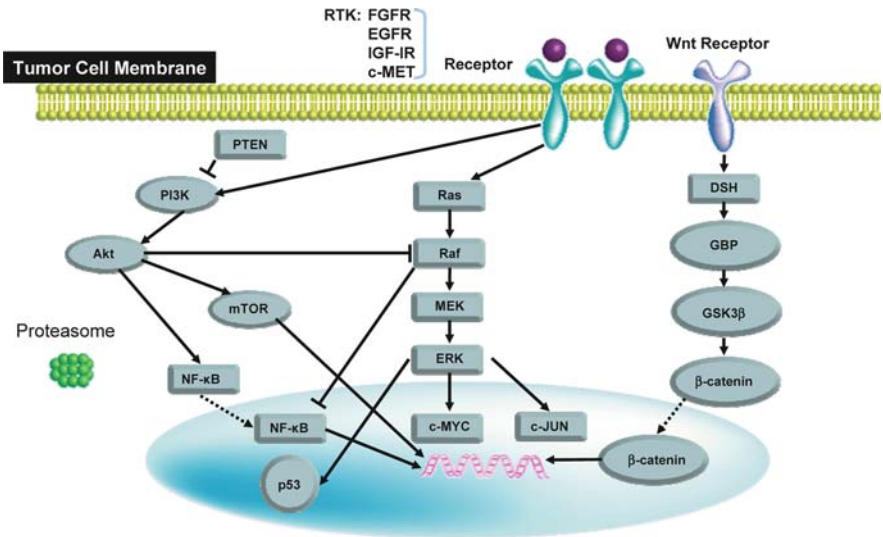

Fig. 1. Growth factor receptor and Wnt receptor pathways.

1. MOLECULAR PATHWAYS

1.1. Growth Factor Receptors

Growth factor receptors, such as epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), stem cell growth factor receptor (c-KIT), and the hepatocyte growth factor receptor (MET), bind to their ligands and form receptor dimers. Dimerization initiates autophosphorylation of intracellular receptor domains, which then leads to the phosphorylation of intracellular second-messenger proteins *(1, 2)*.

Mutations in growth factor receptor pathways have been found in tumors from patients with HCC. EGFR mRNA is upregulated in tissue samples from patients with HCC. Likewise, an increase in the amount of EGFR ligands that can activate these receptors, such as transforming growth factor alpha (TGF-α), has been found in HCC cell lines. Constitutively, activated growth factor receptors are another type of mutation associated with hepatocarcinogenesis; thus, even in the absence of ligand, the pathway can be activated *(3)*.

1.2. Ras/Raf/MAP/ERK Pathway

When Ras, a GTPase, is covalently bound to a prenyl group, it is localized to and associates with the plasma membrane, where it couples with extracellular growth factor receptors *(4, 5)*. Binding of the extracellular receptor to the ligand induces receptor homodimerization or heterodimerization and autophosphorylation of intracellular receptor domains. Ras then undergoes a conformational change from an inactivated state, Ras-GDP, to an active state, Ras-GTP *(4, 6)*. The conformational change induces a series of intracellular phosphorylations: Ras phosphorylates Raf, which then phosphorylates MAP, and MAP phosphorylates numerous proteins, including ERK and several transcription factors, such as c-myc and c-jun *(4, 6, 7)*. Phosphorylated ERK translocates into the nucleus and activates several transcription factors *(4, 7)*.

The Ras/Raf/MAP/ERK pathway has been implicated in numerous cancer types; 15–30% of all cancers have Ras mutations *(7–9)*. Some cancer types, such as HCC, demonstrate an even greater vulnerability to mutations in this pathway. Tumor biopsies from patients with HCC were analyzed for *c-raf-1* gene and Raf-1 protein expression; the overexpression of the *c-raf-1* gene was observed in 50% of samples and overactivity of Raf-1 was observed in 100% of samples *(10)*. Furthermore, Raf mutations are frequently associated with hyperphosphorylated downstream effectors. Raf mutations associated with cancer were transfected into cell lines, and the majority of the various Raf mutations (82%) had hyperphosphorylated ERK in the transfected cells *(11)*.

The Ras pathway can also be controlled through inhibitors such as RASSF1A and NORE1A. The amount of these inhibitors is associated with the presence of HCC and disease status. RASSF1A was significantly decreased in the liver samples from patients with HCC (both good and poor prognosis) compared with liver samples from healthy patients. NORE1A, on the other hand, was decreased only in liver samples from patients with HCC and poor prognosis; there was no difference between the amount of NORE1A in the liver samples of healthy patients and patients with HCC and good prognosis, suggesting NORE1A may be a target to prevent worsening of HCC *(12)*.

1.3. JAK/STAT Pathway

When growth factor receptors bind to their ligands, the receptors undergo dimerization and autophosphorylation of the intracellular cytoplasmic domains. JAK proteins are phosphorylated and JAK phosphorylates the cytoplasmic protein STAT. Phosphorylated STAT forms homodimers, and the STAT dimer translocates into the nucleus and acts as a transcription factor. STAT dimers are quickly inactivated by inhibitors of STAT, suppressors of cytokine signaling (SOCS) *(13)*.

In tumors from patients with HCC, JAK and STAT were hyperphosphorylated; the phosphorylation levels of JAK1, JAK2, STAT3, and STAT5 were significantly higher in the liver samples from patients with HCC than in patients with normal livers. Mutations were found in many of the STAT inhibitors, such as SOCS1, SOCS2, and SOC3 *(12)*.

1.4. The PI3K/Akt/mTOR Pathway

PI3K associates with the intracellular domain of many growth factor receptors. Upon binding of ligands to a growth factor receptor, the growth factor receptors form dimers, and intracellular domains of the growth factor receptors are phosphorylated. The PI3K/Akt/mTOR pathway is activated. PI3K phosphorylates phophatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) *(6)*. The accumulation of PIP3 induces a series of intracellular events, including the activation of Akt, and Akt in turn phosphorylates mTOR, a serine/threonine kinase *(13–15)*. Activated mTOR promotes the expression of *c-myc, cyclin D*, and other genes involved in cell proliferation and angiogenesis. Mutations that induce the constitutive activation of Akt, which then increase the activity of mTOR, have been found in several types of cancers *(1)*.

1.5. Wnt/β-Catenin

Wnts are secreted glycoproteins that bind to the extracellular receptors frizzled, LRP5, and LRP6. In the absence of the ligand, some of the intracellular protein β-catenin forms a complex with E-cadherin, a complex responsible for cell-cell adhesion. β-Catenin also forms a complex with GSKβ, which is then degraded by a proteasome. Upon binding of Wnt to extracellular receptors, a downstream effector phosphorylates β-catenin. Phosphorylated β-catenin dissociates from many of the protein complexes, and this induces other cellular activities. When β-catenin dissociates from E-cadherin, cell motility is enhanced. When β-catenin is phosphorylated and free from the GSKβ complex, it translocates into the nucleus and acts as a coactivator to stimulate the transcription of genes, such as *c-myc, c-jun,* and *cyclin D2 (1, 3)*.

1.6. Transcription Factors

Transcription factors that induce the transcription of genes that promote cell division, cell survival, angiogenesis, or that inhibit apoptosis can lead to cancer. Nuclear factor-kappa B (NF-κB) is a transcription factor known to be associated with hepatocarcinogenesis that induces the transcription of anti-apoptotic genes *(1)*.

In the inactive form, NF-κB remains in the cytoplasm and is bound to an inhibitory protein, inhibitory kappa B (IκB). There are several mechanisms that can remove IκB and, in turn, activate NF-κB. For example, inhibitor kappa kinase can phosphorylate IκB, and phosphorylated IκB dissociates from NF-κB. IκB can also be removed by a specialized proteasome-degradation pathway. When no longer associated with IκB, NF-κB translocates into the nucleus and functions as a transcription factor *(6, 16)*. The PI3K/Akt pathway can also activate NF-κB; Akt phosphorylates numerous proteins and can also activate NF-κB *(17)*. Constitutively, active NF-κB has been found in some forms of cancer and has been associated with hepatocarcinogenesis *(1, 18)*.

1.7. Proteasome

Cells remove intracellular proteins by a specialized proteasome-degradation pathway. The protein to be degraded is covalently linked to ubiquitin molecules by ubiquitin ligases. The chain of ubiquitin molecules bound to the protein 'tags' the protein for a special degradation pathway, and the proteasome destroys the ubiquitinated protein.

Proteasomes are essential for the regulation of cellular activities, such as cell division and gene expression. Cyclins, protein regulators of the cell cycle, are degraded at key steps by proteasomes; in this manner, the

cell progresses to the next stage of the cell cycle. Gene expression is also controlled by proteasomes. For example, proteasomes degrade IκB, an inhibitor of NF-κB. In this manner, NF-κB is activated and can then function as a transcription factor *(6, 19)*.

1.8. VEGF and PDGF

Activation of vascular endothelial growth factor receptors (VEGFRs), including VEGFR1 (FLT-1), VEGFR2 (FLK1-KDR), and VEGFR3 (FLT4), or platelet-derived growth factor receptors (PDGFR)-α or -β promotes angiogenesis. Activation of VEGFR2 on endothelial cells in particular promotes a strong mitogenic, survival, and angiogenic signal. The intracellular molecular pathway is similar to that of growth factor receptors. Upon binding to the ligand, VEGFR forms dimers and activates the intracellular Ras/Raf/MAP/ERK and PI3/Akt/mTOR pathways (Fig. 2) *(3)*.

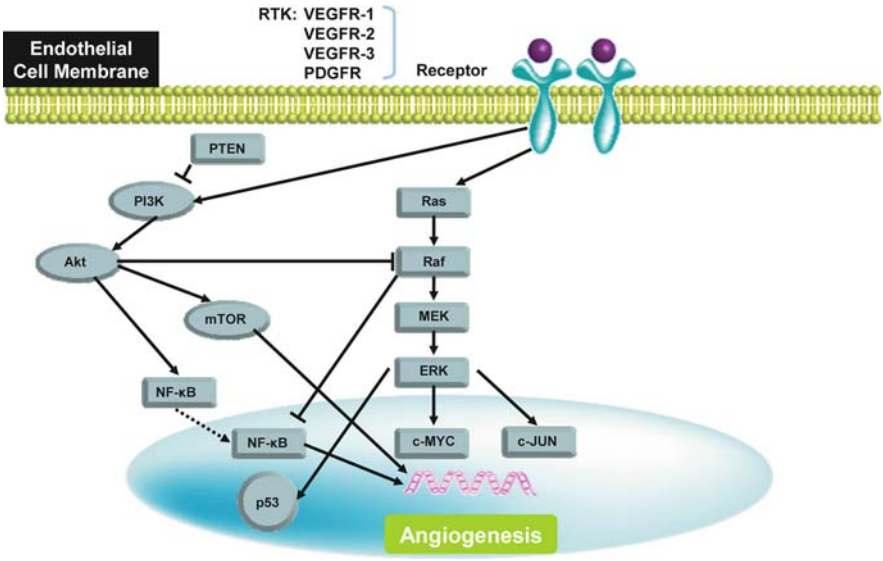

Fig. 2. Signaling pathways for VEGFR and PDGFR.

VEGF levels have been found to correlate with the amount of angiogenesis and poor prognosis. When tumor samples from patients with HCC were collected and analyzed, VEGF levels correlated with the amount of angiogenesis. Furthermore, higher preoperative VEGF serum levels correlated with shorter disease-free survival and overall survival *(20)*.

1.9. Extracellular Matrix Changes

Changes in the extracellular matrix (ECM) can lead to tumor invasion, metastasis, and the worsening of HCC. HCC tissue has been found in association with overexpression of several types of matrix metalloproteinase (MMP) enzymes, such as MMP-2, MMP-7, and MMP-9, which digest ECM proteins.

In addition, changes in the expression of integrins, receptors that mediate cell–cell and cell–ECM adhesion, have been found in tissue from patients with many types of cancer, including HCC *(1, 19, 21)*.

1.10. Apoptosis

Anti-apoptotic transcription factors activated by the second-messenger systems, such as the activation of growth factor receptors and the Ras/RAF/MEK/ERK pathway *(13)*, can lead to inhibition of apoptosis.

Another protein that is essential to prevent cancer is the *p53* gene. This protein can induce apoptosis *(22)*. Similarly, *p53* plays an essential role in HCC; *p53* gene mutations are associated with 30–50% of biopsies from patients with HCC. Furthermore, correlations between p53 mutations and shorter survival time have been observed *(19, 23, 24)*.

1.11. Challenges to the Modification of These Pathways for the Treatment of HCC

Although researchers now understand many of these molecular pathways and have identified factors that could induce mutations that lead to intracellular changes, several challenges still exist. HCC is molecularly heterogeneous; in other words, the underlying pathology that leads to the development of HCC may be different from patient to patient, and a pharmacologic agent may only exhibit efficacy in a subgroup of patients. Another challenge is that some mutations with a constitutively active protein potentiate not one but several intracellular pathways. For example, a mutation in a growth factor receptor that causes constitutive activation of the EGFR could potentially overactivate the Ras/Raf/MAP/ERK, PI3K/AKt/mTOR, and JAK/STAT pathways. If a pharmacologic agent targets either the receptor or the point of signal transduction, then treatment necessitates a therapeutic agent that targets several pathways or the use of a combination of agents that target several pathways. Another challenge is that there is cross talk among many of these intracellular pathways. Therefore, successful modification of one pathway could lead to an increase or decrease in the activity of another pathway or even cause changes that lead to resistance of the pharmacologic agent *(1)*.

2. RATIONAL THERAPIES

2.1. Targeting Growth Factor Receptors

Inhibiting or preventing the activation of growth factor receptors has been a strategy to prevent activation of intracellular molecular pathways, such as Ras/Raf/MEK/ERK and P13/Akt/mTOR. There are several pharmacologic agents in development that target one growth factor receptor in particular—the EGFR. The two strategies that target the EGFR include antibodies that bind to an extracellular domain of the receptor and EGFR tyrosine kinase inhibitors.

Monoclonal anti-EGFR antibodies include cetuximab (Erbitux), a monoclonal IgG1 chimeric antibody, and panitumumab (Vectibix), a monoclonal IgG2 antibody. Both of these antibodies bind to a ligand-binding site on the extracellular domain of the EGFR and reduce activation of the EGFR *(17, 25)*. Although both cetuximab and panitumumab are antibodies, they have differing mechanisms of action. Cetuximab has been proposed to stimulate antibody-dependent cell-mediated cytotoxicity, whereas panitumumab is believed not to activate antibody-dependent cell-mediated cytotoxicity *(17, 25, 26)*. Another difference is the final destination of the receptors that bind to the antibodies. Cetuximab binds to receptors and stimulates endocytosis, but the antibodies are later returned to the cell surface, whereas receptors bound to panitumumab undergo endocytosis but are then degraded *(17, 25)*. Other monoclonal anti-EGFR antibodies in development include MDX-447, nimotuzumab, mAb806, and matuzumab *(17)*.

Gefitinib (Iressa) and erlotinib (Tarceva) are EGFR tyrosine kinase inhibitors. These agents compete with the ATP intracellular domain of EGFR inhibitors and prevent activation of the intracellular cascade *(25)*. Other EGFR tyrosine kinase inhibitors in clinical development include EKB-569, PKI-166, and canertinib *(17)*. Tyrosine kinase inhibitors that target EGFR in addition to another receptor are also in development; these include both lapatinib (Tykerb) and BMS-500626, which targets EGFR and erbB2.

Because some of the agents that target EGFR, such as gefitinib, erlotinib, and cetuximab, are approved for other cancer types, agents that similarly target EGFR are thought to have the potential to treat HCC. However, agents that target EGFR have mixed results in the treatment of other tumor types. Some patients do not respond to anti-EGFR therapy and other patients who initially respond develop resistance *(27)*. Thus, many current and recently completed clinical trials evaluate the efficacy and safety of anti-EGFR pharmacologic agents alone or in combination for patients with HCC *(1)*.

In addition to pharmacologic agents that target EGFR, there are also agents in development that target another growth factor receptor, insulin-like growth factor-1 receptor (IGF-1R). Pharmacologic agents that target

IGF-1R include anti-IGF-1R antibodies (e.g., CP-751, A12, scFv-Fc, and AVE-1642) and IGF-1R inhibitors (e.g., NVP-AEW541, NVP-ADW742, BMS-536924, cyclolignans, and INSM-18, an agent that targets IGF-1R and HER2) *(13)*.

2.2. Targeting Ras/Raf/MAP/ERK

Farnesyl transferase inhibitors (FTIs) inhibit Ras and the downstream effector enzymes. FTIs target farnesyl transferase, which adds fatty acids to Ras post-translation. Without the addition of fatty acids to Ras, Ras would not be localized to the plasma membrane, and activated growth factor receptors would be incapable of signal transduction. FTIs in development include lonafarnib, tipifarnib, R115777, BMS214622, and UDF-1 (manumycin) *(6, 19)*.

MEK inhibitors that target the *Ras/Raf/MAP/ERK* pathway slightly downstream, such as AZD6244, CI-1040, and PD184161 *(28)*, are in development. Phase II clinical trials to evaluate AZD6244 in patients with HCC are currently recruiting patients.

2.3. Targeting PI3K/Akt/mTOR

Several pharmacologic agents targeting the *PI3K/Akt/mTOR* pathway have been developed. Although some of the agents that inhibited the activity of PI3K (e.g., wortmannin and LY294002) were initially promising in tumor xenograft models, later studies demonstrated that they would not be appropriate as clinical agents because their pharmacokinetic properties were not favorable *(29)*. Other therapeutic agents, such as alkylphospholipid perifosine, target Akt *(30)*. Perifosine is currently undergoing clinical evaluation for efficacy for several tumor types.

There are many agents in development that block the downstream effectors PI3K and Akt proteins, mTOR. The mTOR inhibitors in development include everolimus, temsirolimus, and sirolimus *(1, 19)*. Everolimus is currently approved for another tumor type. There are several phase I/II trials evaluating an mTOR inhibitor in patients with HCC that are in the process of recruiting patients.

2.4. Targeting Wnt/β-Catenin

To prevent activation of the Wnt/β-catenin pathway, monoclonal antibodies that bind to Wnt-1 and Wnt-2 have been developed *(1, 31–33)*. Another agent in development is ICG-001, a drug that disrupts the interaction between β-catenin and the transcription regulator CREB-binding protein *(30, 34)*. Although anti-Wnt antibodies and agents that disrupt activity

of the downstream Wnt effector, β-catenin, promote apoptosis in cancer cell lines, these agents are still in preclinical development *(1, 31, 34–36)*.

2.5. Proteasome Inhibitors

A proteasome inhibitor in development is bortezomib (Velcade). In preclinical studies, proteasome inhibitors demonstrated efficacy when delivered with other agents; bortezomib was given as a pretreatment to cells followed by a tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) *(37)*. Apoptosis was induced only in HCC cells, whereas non-HCC hepatocytes did not exhibit apoptosis *(37)*. Agents that target growth factor receptors, the Ras/Raf/MEK/ERK pathway, mTOR, and proteasomes, are shown in Fig. 3.

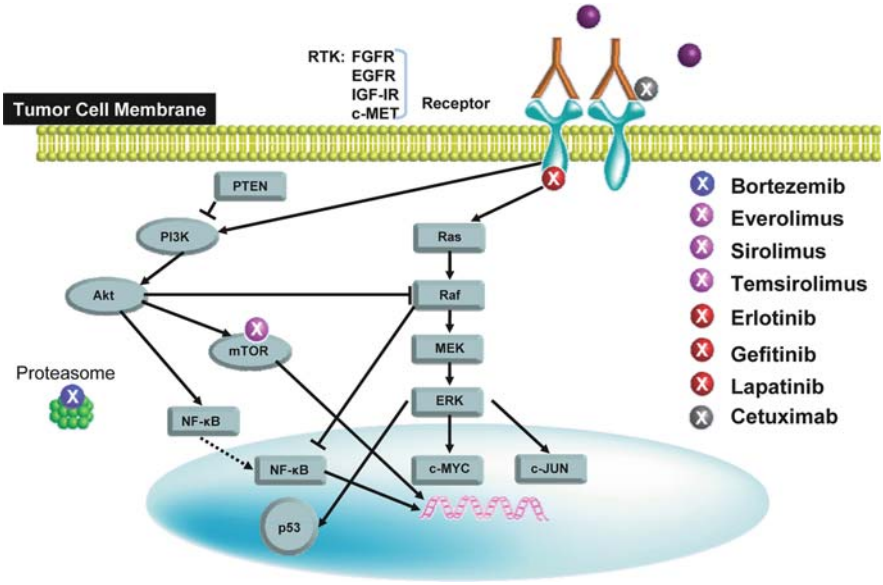

Fig. 3. Molecular targets of growth factor receptor and intracellular pathways.

2.6. Targeting Transcription Factors

The enzyme inhibitor kappa kinase has been targeted with molecularly targeted agents that inhibit this particular enzyme and includes agents such as quinazoline analogs and β-carbolin. By inhibiting inhibitor kappa kinase, IκB will not be phosphorylated and NF-κB will not be tagged for proteasome degradation *(1)*. Although kappa kinase inhibitors have not yet been evaluated in clinical trials for patients with HCC, there are active phase II trials

assessing the efficacy of a pharmacologic agent that inhibits proteasomes. Proteasomes regulate the activity of several proteins, including NF-κB.

2.7. Targeting VEGFR and PDGFR

Because VEGFR and PDGFR stimulate proangiogenic pathways, pharmacologic agents that target these receptors can inhibit this process. Pharmaceuticals in development that target the VEGFR include agents that remove the ligand, such as VEGF antibodies and VEGFR kinase inhibitors. Agents that remove the ligand include bevacizumab and HuMV833, anti-VEGF antibodies, and VEGF-TRAP (aflibercept), a fusion protein that binds circulating VEGF *(19)*. There are phase I clinical trials in progress to assess VEGF-TRAP, (aflibercept) in patients with relapsed or refractory solid tumors. VEGFR inhibitors in development include brevanib and cediranib, VEGFR-2 kinase inhibitors, and vetalanib, a KIT and VEGR-1, -2, and -3 inhibitors.

Drugs that target PDGFR are also in development. TSU-68 is a tyrosine kinase inhibitor of PDGFR-β and BEGFR-2. Agents that inhibit the angiogenic pathways are shown in Fig. 4.

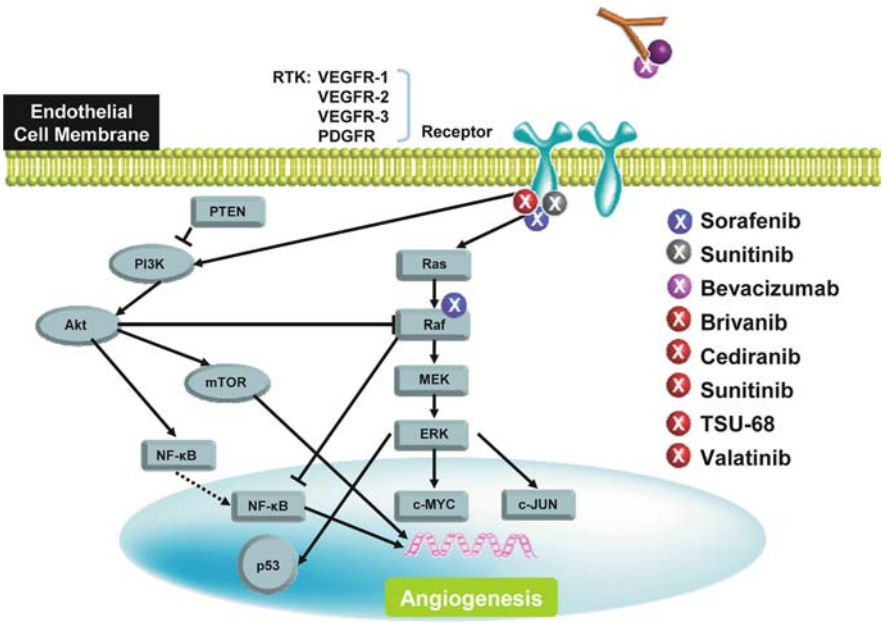

Fig. 4. Antiangiogenic targets of molecularly targeted agents.

2.8. Apoptosis

Mutations in the *p53* gene are associated with cancer and the development of HCC. The correct functioning of this gene and protein is essential for the regulation of the cell cycle and the initiation of apoptosis. Currently, there are systems in development that are a construct of the adenovirus and the *p53* gene. An ongoing phase I clinical trial is assessing Ad5CMV-p53 for patients with HCC.

There are other pharmacologic agents in development that target genes in the apoptotic pathway, such as LY2181308, antisense oligonucleotides to the protein survivin. The protein survivin inhibits activation of caspase-9. In preclinical studies, coadministration of survivin and chemotherapeutic agents increased the activity of caspases and promoted apoptosis in cancer cell lines and inhibited tumor growth in xenografts *(38)*. A phase I/II clinical trial to assess LY2181308 in patients with HCC has not yet begun recruiting patients but is currently registered.

2.9. Metalloproteinase Inhibitors

MMPs degrade and digest extracellular matrix proteins, changes associated with metastasis. Pharmacologic agents that inhibit one or several types of MMPs include metastat, neovastat, batimastat, marimastat, BAY12-9566, AG-3340, OPB-3206, KBR07785, and KBR-8301. These agents have been tested in solid tumors and were found to have low response rates. Researchers have suggested that they may be more efficacious at earlier stages of disease, such as in patients at risk for developing HCC rather than patients with advanced HCC *(19)*.

2.10. Multitargeted Kinase Inhibitors

The two multitargeted kinase agents that are most advanced in clinical development are sorafenib and sunitinib.

Sorafenib (Nexavar) inhibits the Ras/Raf/MAP/ERK pathway, VEGFR-2 and -3, PDGFR-β, KIT, RET, and Flt-3 receptor tyrosine kinases *(39–41)*. In addition to blocking multiple pathways, sorafenib is the first systemic agent that has provided clinical benefit to patients with HCC. In a phase III trial (SHARP trial), 602 patients predominantly from Europe, Australia, and the United States and diagnosed with advanced HCC were randomized to receive either placebo or sorafenib at 400 mg twice a day. Patients in the placebo arm had an overall survival of 7.9 months, whereas patients in the sorafenib arm had an overall survival of 10.9 months ($P = 0.00058$) *(42)*. Sorafenib was generally well tolerated. Drug-related adverse events reported by 10% of patients or more included diarrhea, hand–foot skin reaction, anorexia, alopecia, and nausea *(43)*. Based on the improvements in health outcomes, such as overall survival, demonstrated in patients administered

sorafenib in this phase III trial, sorafenib was granted FDA approval. Sorafenib is the first molecularly targeted agent to reach the clinic for the treatment of HCC.

Although this trial demonstrated that sorafenib significantly improved overall survival, it should be noted that 96% of the patients in this trial were Child–Pugh class A. Thus, more studies are needed to evaluate the efficacy and safety of sorafenib in patients with Child–Pugh classes B and C *(30)*. Consensus guidelines by the National Comprehensive Cancer Network (NCCN) reflect the need for future studies to assess the safety of sorafenib in patients with Child–Pugh class B and C status. The guidelines suggest that patients with inoperable HCC and either Child–Pugh class A or B status receive sorafenib, with the caveat that patients with Child–Pugh class B status be administered the drug with caution, because there are only limited safety data available with Child–Pugh class B status *(44)*.

The benefit of sorafenib has also been validated in another large ($n = 226$) randomized, placebo-controlled, phase III trial. This trial was conducted in the Asia-Pacific region and many patients (73.0%) had hepatitis B virus. Overall survival significantly improved in patients receiving sorafenib ($P = 0.014$); patients in the placebo arm had a median overall survival of 4.2 months, whereas patients in the sorafenib arm had a median overall survival of 6.5 months. The most common adverse events included hand–foot skin reaction, diarrhea, alopecia, and fatigue *(45)*. While there was a significant survival benefit derived from sorafenib therapy, it should be noted that the overall survival was lower in this trial, including the placebo population compared with the SHARP trial *(46, 47)*.

Because sorafenib is the first agent to reach the clinic and improve overall survival in patients with HCC, clinical trials are currently in progress to evaluate whether the benefits of sorafenib can be improved. Strategies under evaluation include sorafenib in the adjuvant setting, post-transarterial chemoembolization (TACE), sorafenib in combination with other agents, and dose escalation.

Sunitinib (Sutent) inhibits VEGFR-1 and -2; PDGFR-α and -β; stem cell factor receptor c-KLT; and the FLT3 and RET kinases *(2)*. In a phase II trial, sunitinib was administered to patients with unresectable HCC. In two recent phase II clinical trials, sunitinib appeared to provide benefit. The primary end point was overall response rate, as assessed by RECIST criteria. The most common adverse events were diarrhea, anorexia, nausea, and asthenia. Other multitargeted kinase inhibitors in development for HCC include brivanib (AEE788)—an inhibitor of EGFR, erbB-2, and VEGFR-2 *(19)*; vandetanib—a VEGFR and EGFR tyrosine kinase inhibitor; ABT-869—a VEGFR and PDGFR tyrosine kinase inhibitor; and vatalanib—a VEGFR, PDGFR, and c-KIT tyrosine kinase inhibitor *(13)*. Dual-mechanism agents, such as PI-88, a heparin sulfate antagonist and VEGF, FGF-1, and FGF-2 signaling inhibitor, are also in development.

The mechanisms of action of the various molecularly targeted agents are summarized in Table 1. Clinical trials with single molecularly targeted agents in patients with HCC are summarized in Table 2.

Table 1
Overall Mechanism of Action of Pharmacologic Agents

	Antiangiogenic targets			*Antiproliferative targets*		
Agent	*VEGF*	*VEGFR*	*PDGFR*	*EGF*	*EGFR*	*mTOR*
ABT-869*		•	•			
Bevacizumab[a] (Avastin)	•					
BMS-599626					•	
Brivanib (BMS-582664)	•					
Cediranib (Recentin)	•					
Cetuximab[b] (Erbitux)					•	
Erlotinib[c] (Tarceva)					•	
Everolimus (Certican)						•
Gefitinib[d] (Iressa)					•	
IMC-112B		•				
Lapatinib[e] (Tykerb)					•	
Panitumumab[f] (Vectibex)				•		
Sirolimus (Rapamune)						•
Sorafenib*[g] (Nexavar)		•	•			
Sunitinib*[h] (Sutent)		•	•			
Temsirolimus[i] (Torisel)						•
TSU-68	•	•	•			
Vatalanib (PTK787)		•	•			
Vandetanib* (Zactima)		•			•	

∗Multitargeted tyrosine kinase inhibitor.

[a]Approved for metastatic breast cancer, metastatic colorectal cancer, and non-squamous, non-small cell lung cancer;

[b]Approved for squamous cell carcinoma of the head and neck and EGFR-expressing metastatic colorectal carcinoma;

[c]Approved for non-small cell lung cancer and pancreatic cancer;

[d]Approved for locally advanced or metastatic non-small cell lung cancer after failure of both platinum-based and docetaxel chemotherapies who are benefiting or have benefited from gefitinib;

[e]Approved for HER2 positive breast cancer;

[f]Approved for EGFR-expressing metastatic colorectal carcinoma;

[g]Approved for HCC and renal cell carcinoma;

[h]Approved for renal cell carcinoma and gastrointestinal stromal tumor after disease progression on or intolerance to imatinib;

[i]Approved for advanced renal cell carcinoma.

Table 2
Single Agents Evaluated or Undergoing Evaluation in Clinical Trials

Pharmacologic agent	*Mechanism of action*	*Phase*	*N*	*End points*
ABT-869	Multitargeted TKI; VEGFR, PDGF	II	40	PFS, ORR, safety
AZD-6244	MEK inhibitor	II	44	ORR, TTP, PFS, safety, OS
Bortezomib	Apoptosis inducer; 26S proteasome inhibitor	II	22	Safety, MTD
Bevacizumab	mAb; anti-VEGF	II	30	24 evaluable, PR = 3, SD = 13 (including >16 weeks in 7 patients)
Bevacizumab	mAb; anti-VEGF	II	46	PFS, safety
Brivanib	Multitargeted TKI; VEGFR-2, VEGFR-3, FGFR-1, FGFR-2	II	100	6-month PFS, ORR, OS
Cediranib	Multitargeted TKI; VEGFR-1, VEGFR-2, VEGFR-3	II	44	6-month OS, ORR, safety
Cetuximab	mAb; anti-EGFR	II	32	27 evaluable, SD (= 8 weeks) 44.4%, TTP = 8 weeks
Dasatinib	Multitargeted kinase inhibitor; BCR/ABL	II	41	PFS, ORR, PFS, OS, safety
Erlotinib	TKI; EGFR inhibitor	II	80	PFS, OS, ORR, safety
Erlotinib	TKI; EGFR inhibitor	II	40	PFS, OS, ORR, safety
Everolimus	Rapamycin analogue; mTOR inhibitor	I/II	134	MTD, DCR
Gefitinib	TKI; EGFR inhibitor	II	31	ORR, safety
IDN-6556	Caspase inhibitor	II	100	Efficacy, safety
IMC-1121B	mAb; anti-VEGFR-2	II	40	PFS, TTP, ORR, safety

(Continued)

Table 2
(Continued)

Pharmacologic agent	*Mechanism of action*	*Phase*	*N*	*End points*
Lapatinib	Dual TKI; EGFR and HER-2/neu	II	34	ORR, OS, safety
PI-88	Heparanase inhibitor; VEGF, FGF inhibitor	II/III	343	Recurrence rate, RFS, OS
Sorafenib	Multitargeted TKI; Raf, PDGFR-b, VEGFR-2/-3, KIT, Flt-3	III	602	OS, TTSP, TTP, safety vs placebo
Sunitinib	Multitargeted TKI; VEGFR-2, PDGFR-b, KIT, Flt-3	II	34	PFS, ORR, OS
TSU-68	Multitargeted TKI; VEGFR, PDGFR, FGFR	I/II	N/A	Safety/feasibility
Vandetanib	TKI; VEGFR	II	75	4-month stabilization, ORR, PFS, OS, safety

DCR = disease control rate; MTD = maximum tolerated dose; ORR = overall response rate; OS = overall survival; PFS = progression-free survival; RFP = relapse-free survival SD = stable disease; TTP = time to progression; TTSP = time to symptomatic progression

2.11. Polypharmacy

There are two strategies for the use of polypharmacy in HCC. Some agents demonstrate low to moderate efficacy that could be enhanced if given in combination with another agent. For example, clinical trials are currently evaluating anti-EGFR agents in combination with other molecularly targeted agents. Cetuximab decreases the transport of EGFR to the nucleus and the corresponding activation of DNA enzymes. Use of cetuximab has been proposed as a means of making cells more susceptible to chemotherapeutic agents; thus, cetuximab and a chemotherapeutic agent could be used in combination *(17)*. Another strategy is the inhibition of two pathways simultaneously—for example, using a multitargeted kinase inhibitor and an mTOR inhibitor. Current trials using polypharmacy to treat HCC are summarized in Table 3.

During the past 30 years, a systemic therapy that could improve survival outcomes of patients with advanced HCC remained an unmet need. A meta-analysis of therapies used in randomized, controlled clinical trials

Table 3
Combination Trials with Molecularly Targeted Agents

Treatment	*Phase*	*N*	*End points*
Bevacizumab + capecitabine	II	45	ORR, DCR, median OS, PFS
Bevacizumab + capecitabine	II	100	PFS rate at 27 weeks
Bevacizumab + capecitabine + oxaliplatin	II	30	DCR, PFS
Bevacizumab + chemoembolization	II	30	PFS, safety, ORR
Bevacizumab + dexamethasone+ floxuridine	II	55	ORR, safety
Bortezomib + doxorubicin	II	40	ORR, OS, TTP, safety
Bevacizumab + erlotinib	II	29	Tumor response rate
Bevacizumab + erlotinib	II	21	PFS rate at 27 weeks, tumor response rate
Bevacizumab + erlotinib	II	40	PFS rate at 16 weeks, ORR, OS
Cetuximab + gemcitabine + oxaliplatin	II	43	ORR, DCR, safety
Bevacizumab + oxaliplatin + gemcitabine	II	30	TTP, ORR, safety
Bevacizumab + TACE	II	30	Neovessel formation, tumor progression
Bevacizumab + TACE	II	40	1-year tumor response, OS, safety
Celecoxib + erlotinib as adjuvant	I/II	50	OS, DFS, safety, MTD
Gefitinib adjuvant to resection	II	40	Identification of biomarkers, RFS

(Continued)

Table 3
(Continued)

Treatment	*Phase*	*N*	*End points*
PI-88 adjuvant to curative therapy	III	N/A	Tumor nonrecurrence, time to recurrence, OS
Sorafenib + doxorubicin	II	96	TTP, safety
Sorafenib + UFUR	II	50	PFS, 6-month PFS, ORR, OS
Sorafenib + TACE with doxorubicin	I	N/A	MTD, DLT

DCR = disease control rate; DLT = dose-limiting toxiciy; MTD = maximum tolerated dose; ORR = overall response rate; OS = overall survival; PFS = progression-free survival; RFP = relapse-free survival TACE = transarterial chemoembolization; TTP = time to progression

published from 2002 to 2005 concluded that systemic therapies did not provide a survival advantage to patients with advanced HCC *(48)*. Sorafenib is the first molecularly targeted pharmacologic agent to significantly increase overall survival *(49)*. Several other molecularly targeted pharmacologic agents are under evaluation in clinical trials, either as stand-alone drugs (Table 2) or in combination with other pharmacologic agents (Table 3). The results from several clinical trials that evaluated targeted therapies as a stand-alone drug or in combination therapy for patients with HCC were reported at the American Society of Clinical Oncology (ASCO) 2008 meeting and are summarized in Table 4. Over the next few years, it is expected that the knowledge gained from advances in molecular biology will finally translate to real victories in the war against cancer and provide pharmacologic agents that can provide benefit to the patient, such as improved survival, better management of symptoms, and preservation of quality of life.

3. UNANSWERED QUESTIONS

For the first time, molecularly targeted treatments that provide clinical benefit to patients with cancer are available. Many more are in development and are being assessed in clinical trials. These profound accomplishments should not be underestimated. Nonetheless, if investigators can provide answers to these unanswered questions over the next few years, these agents could provide even more benefits to patients.

Table 4
Studies with Targeted Agents Reported at the American Society of Clinical Oncology (ASCO) 2008 Meeting

Treatment	*N*	*Phase*	*End points*
Bevacizumab	48	II	Contrast-enhanced ultrasonography parameters, CT scans
NGR-hTNF	16	II	PFS
Sorafenib	226	III	OS, TTP, FSHI8, DCR, safety
Sunitinib	34	II	PFS, angiogenic markers
TSU-68	35	I/II	ORR, DCR
Bevacizumab + capecitabine	45	II	ORR, PFS, OS
Bevacizumab + erlotinib	34	II	RR, EGFR expression, EGFR mutation status, VEGF levels, VEGF-1 and -2 expression
Bortezomib + doxorubicin	39	II	RR, OS, PFS
Cetuximab, oxaliplatin, capecitabine	25	II	OR, TTP, OS
Sorafenib + tegafur/uracil	40	II	PFS

DCR = disease control rate; FSHI8 = time to symptom progression; ORR = overall response rate; OS = overall survival; PFS = progression-free survival; RR = response rate; TTP = time to progression

3.1. Can Treatment Outcomes Be Optimized by Matching the Drug with the Patient?

One interesting area of research is the use of biomarkers to predict sensitivity of a patient to a particular drug. In a phase II study of sorafenib for patients with advanced HCC, pERK was assessed as a biomarker *(50)*. First, pERK levels were measured in pretreatment biopsies. In the post-treatment analysis, researchers observed a correlation between pretreatment pERK levels and sensitivity to sorafenib; patients with high pERK had significantly longer time to progression than patients with low pERK ($P = 0.00034$). It will be interesting to see if the findings from this phase II trial can be replicated in a larger phase III trial or if other molecularly targeted agents can

demonstrate a correlation between a biologic marker (such as baseline tumor levels of pERK, pAKT, EGFR, or plasma levels of the angiogenic cytokine VEGF-A) and clinical benefit. Many clinical trials in progress are assessing the role of biomarkers to predict prognosis and sensitivity to medication in their trials. Perhaps there will be a time when, after the analysis of a patient biopsy, the best therapeutic option can be matched with the patient's biopsy results.

In the future, correlating the appearance and severity of an adverse event after the initiation of a drug with its efficacy may be useful for deciding whether to continue with a particular drug or not. Many researchers have observed a correlation between the presence and/or severity of skin toxicity—an adverse event associated with agents that target EGFR—and efficacy of the medication as assessed by response rate, progression-free survival, and overall survival *(25, 51, 52)*. Similarly, through the pooled analysis of four sorafenib clinical trials, investigators have proposed that adverse events, such as the incidence of diarrhea and hand–foot skin reaction, correlate with progression-free survival *(53)*. However, minimizing adverse events in patients administered a particular drug should still be a goal, because patients may need to have dose reductions or discontinue with a drug because of adverse events.

3.2. Can the Efficacy of Current Treatments Be Improved?

The potential to improve the efficacy of a molecularly targeted agent should remain a goal, even after a drug is granted approval. Current strategies under exploration include determining the optimal therapeutic index and polypharmacy.

One strategy to find the optimal therapeutic index includes dose escalation; provided that an increase in adverse events does not occur, efficacy could be improved. In a phase II trial of sorafenib for patients with renal cell cancer, patients were allowed to dose escalate while researchers evaluated adverse events and efficacy; the majority of patients (91%) were able to escalate to 1200 mg to 1600 mg per day *(54)*. Although this trial included a small sample size and will need to be replicated in a larger population and in patients with RCC, increased dosage may offer an option for further therapeutic benefit. Minimizing the toxicity of the pharmacologic agent is another strategy to improve the therapeutic index. This remains an important need for many of the new molecularly targeted agents. For example, there are no consensus guidelines or clinical trials to evaluate the management of hand–foot skin reaction, an adverse event prevalent in patients treated with sorafenib *(55)*.

Another promising strategy to improve efficacy is polypharmacy. Frequently, several molecular pathways are activated in patients with HCC.

Inhibiting only one pathway may not be sufficient. Inhibition of several pathways may be needed to improve the efficacy of pharmacologic agents. In addition, the likelihood of a patient developing resistance to an agent may be reduced if several pathways are simultaneously inhibited. Current trials are evaluating the efficacy of using a molecularly targeted agent in combination with other pharmaceutical agents.

3.3. Can Populations at Risk for Having HCC Be Identified and Monitored, and Can the Risk Be Decreased?

One population at risk for HCC is patients who were successfully treated for HCC with surgery and are in remission. If patients with HCC present with few tumors and good liver function, resection offers a viable treatment option; 60–70% of patients with HCC who are treated with resection survive for 5 years or more *(56)*. However, despite successful resection, there is a high rate of recurrence. Within 3 years of resection, more than half of patients have recurrence of HCC *(57)*. It will be interesting to evaluate whether treating patients immediately following surgery with a molecularly targeted agent could decrease the percentage of patients with recurrence or prolong the time until recurrence. There are currently clinical trials in progress to assess whether the administration of a molecularly targeted agent such as PI-88 after resection will prevent recurrence of HCC.

Although resection offers a viable treatment option, most patients present with intermediate or advanced HCC, and these patients are ineligible for resection *(56)*. Thus, a strategy to improve patient outcome is to identify patients at risk for HCC at earlier stages. The American Association for the Study of Liver Diseases (AASLD) provides consensus guidelines for the screening and identification of patients at risk for HCC. The guidelines suggest screening patients who are at high risk for HCC, such as patients with hepatitis B infection, virus carriers, and additional risk factors based on ethnicity, sex, and age (e.g., Asian race, men = 40 years) *(58)*. The AASLD consensus guidelines also recommend that at-risk patients be screened with ultrasonography *(58)*.

A more effective strategy is to reduce the likelihood of developing HCC. One strategy currently under evaluation involves the use of vaccines against the hepatitis B virus in populations at high risk for acquiring this virus. If this strategy is successful, it would prevent the development of the hepatitis B virus, and consequently, the development of HCC *(59)*.

3.4. Can Clinically Relevant Trials Be Conducted?

Historically, phase II nonrandomized trials with an end point of tumor regression, as assessed by Response Evaluation Criteria in Solid Tumors

(RECIST) criteria, have been used to evaluate whether a cytotoxic drug does or does not exhibit efficacy in a patient population with a particular tumor type *(60)*. Most cytotoxic agents evaluated in clinical trials, and later given FDA approval, had an overall response (complete and partial) of 20% or more *(60)*. Furthermore, as it is unlikely that partial or complete tumor regression would occur without a patient first receiving a therapeutic agent, nonrandomized trials have been deemed appropriate *(60)*.

The use of clinical trial designs or end points used to assess cytotoxic agents as appropriate to evaluate the novel molecularly targeted agents has been challenged. While cytotoxic agents cause cell death leading to large and measurable changes in tumor growth, some of the molecularly targeted agents act by reducing cell division or angiogenesis and may have minimal to no changes in tumor growth that RECIST criteria may be sensitive enough to measure *(60, 61)*. Consistent with this interpretation, when results from several studies were pooled to determine the overall response rates (complete and partial) for molecularly targeted agents, the overall response rates were low, from 0 to 28%, with only sunitinib achieving an overall response rate >20% *(60)*. Many of these agents with low overall response rates have been approved for several tumor types, and although they have exhibited efficacy (with improvements in overall survival or progression-free survival), overall response rates have not appeared to correlate with efficacy *(60, 62)*. Other researchers note that overall response, although low, does correlate with eventual FDA approval for molecularly targeted agents. Agents with greater response rates other than molecularly targeted agents (3–28%) have been given FDA approval for particular tumor types *(60)*.

Although these data suggest that a molecularly targeted drug evaluated in a clinical trial for a particular tumor type may still exhibit activity, even if the majority of the patients have disease stabilization, caution should be used. It may be difficult to determine whether tumor growth has stabilized because of the effect of the drug or tumor growth dynamics. Randomized trials may be the best way to differentiate whether stable disease is due to drug or tumor growth dynamics *(62, 63)*.

The importance of including a placebo arm has been shown in recent trials. Bevacizumab plus gemcitabine demonstrated efficacy when administered to patients with pancreatic cancer in a nonrandomized, uncontrolled phase II trial on the basis of progression-free survival and 1-year survival, but when compared with a placebo arm in a randomized, placebo-controlled phase III trial, the same pharmacologic agents failed to demonstrate efficacy *(63)*.

Several suggestions to improve clinical trials with the new molecularly targeted agents have been suggested. Researchers have stressed the need for phase II studies to be randomized and include a placebo arm. Most phase II clinical trials evaluating molecularly targeted agents were not random-

ized (only 30% were) *(60)*. Of agents that were randomized, the majority only had arms with alternate doses of the same drug *(60)*. Now that sorafenib has been granted approval and the NCCN guidelines advocate the use of sorafenib in patients with inoperable HCC, some investigators have suggested that sorafenib be included as a comparator arm in future clinical trials *(64)*. In addition, investigators have suggested that clinical trials only include surrogate end points that have demonstrated correlation with overall survival. Although it is understandable that in clinical trials for patients with breast cancer, who may have an expected life span of 8 years, a surrogate end point for life span may be appropriate, perhaps trials that include patients with advanced HCC should use overall survival as an end point. The average patient with nonsurgical HCC (in the West) has a life expectancy of only 17 months *(57)*. Another suggestion is to incorporate end points that assess improvements in quality of life and the management of symptoms *(65)*.

3.5. What Is the Future of Rational Therapies for HCC?

In the next few years, molecularly targeted therapies for HCC will likely be optimized for dosage and scheduling regimens. An individual patient diagnosed with HCC may enter the clinic, and the best drug will be selected based on tumor biomarkers or a pharmacogenomic profile. The patient may begin receiving a molecularly targeted agent either alone or in combination with other molecularly targeted agents or as an adjuvant to surgery; well-designed randomized clinical trials will have evaluated these possibilities. Patients may begin receiving a molecularly targeted agent at a much earlier time point, such as post-surgery. Finally, patients with HCC may no longer have a projected life span of a few months to a few years, but several years and HCC may be managed as a chronic disease similar to breast or colorectal cancer.

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23 Radiation Therapy for Hepatocellular Carcinoma

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CONTENTS

ABSTRACT

Essential to understanding of the role of radiation therapy in hepatocellular carcinoma treatment is the inherent radiosensitivity of the organ compared to that of hepatocellular (HCC) tumors. As a parallel architecture organ, the liver can continue functioning normally if sufficient individual lobules are spared tolerance doses of ionizing radiation. Many factors can determine the extent of radiation cell kill – oxygenation, blood flow, type of radiation delivered and absorbed, just to name a few. The current growing success of radiotherapy in HCC management reflects a sophisticated technical approach – IMRT, IGRT, 3D radiation treatment planning, with a keen understanding of features to exploit utilizing different radioactive isotopes (^{90}Y, ^{131}I) or of the atom itself, e.g., electrons and protons.

Clinical experience of radiotherapy in HCC continues to grow rapidly both in Asia and the rest of the world with increasingly positive outcomes. This chapter presents the fundamental physical, radiobiologic, molecular, and clinical issues that impact the effectiveness of radiation cell killing of

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HCC, followed by an update on state-of-the-art radiotherapy technologies and the latest clinical results.

Key Words: Ionizing radiation; external beam radiation; IMRT; Proton; ^{90}Y; ^{131}I; Microspheres; Brachytherapy; electrons; beta particle

1. OVERVIEW

There are many factors that have over time contributed to the limited use of ionizing radiation in treating hepatocellular carcinoma. Primarily it is due to the fact that delivery of tumorcidal doses of radiation to a tumor will exceed tolerance of the normal surrounding liver. X-rays produce non-discriminatory cell killing in the already diseased liver of HCC patients. In the past, radiation beams could only be delivered in the simplest of geometric arrangements, which could not avoid enough normal liver tissue from x-rays to deliver doses of radiation to control solid tumors. Only in the past 15 years have technological advancements in Radiation Oncology and Diagnostic Radiology allowed for innovative approaches in both external beam and brachytherapy for treatment of liver malignancies. Concurrent with hardware upgrades such as megavoltage linear accelerators, there have been powerful software programs created, which enable conversion of CT or MRI data sets into 3D "virtual" patients. With accurate 3D models of the patient to work from, and estimates in real time of radiation dose deposition within the patient, radiation oncologists can attempt to deliver the higher doses of radiation, which have a chance to control tumor, while sparing the non-malignant hepatocytes. Most solid malignancies are successfully treated with combination therapy, and for years, it has been the desire to apply these approaches to HCC. The technology described is now widely available in all Cancer Centers, and explains in part, why the interest now to treat HCC within multidisciplinary hepatic oncology groups and ongoing clinical trials is increasing. Radiobiologic protectants are now in clinical trials, which may in the future allow for selective sparing of the normal liver cells found within the radiation beam. It is the intent of this chapter to summarize the main techniques historically and currently available in delivering ionizing radiation to HCC and describe interesting new approaches. Clinical experience over the past century suggests radiation dose parameters, above which serious and possibly fatal liver dysfunction occurs. Moreover this occurs when the whole liver (i.e., all functional units of the organ) receives external beam radiation in excess of 30 Gy. State-of-the-art radiotherapy techniques can treat small portions of the liver to cumulative doses of 90 Gy or more as will be discussed later, but the number of patients suitable for this approach is few. Placing radiation directly in the tumor (brachytherapy) holds the promise of success as it can deliver

very large doses of radiation selectively to the tumor (80–300 Gy) while sparing surrounding normal liver parenchyma, which will be reviewed later in the microsphere section.

2. PHYSICS OF RADIATION THERAPY

2.1. External Beam Radiation Therapy

Radiation that is of sufficient energy to cause ionization of cellular contents is used therapeutically, and is either an electromagnetic or particulate energy form. Electromagnetic energy, photons, can be produced naturally by decay of radioactive isotopes (gamma rays) or by an electrical device accelerating electrons, which abruptly stop in a target, releasing energy (x-rays). Particulate energy most commonly is electrons (charge –1, mass = 0.511 MeV), but others in limited use for cancer therapy include protons (charge +1, mass=2,000 × electrons), alpha particles (helium ions), and neutrons (same mass as proton, no charge).

External beam radiotherapy is what is most commonly employed for nearly all cancers, using x-rays. Photons, which are discrete packets of electromagnetic energy, cause cell damage or cell death by apoptosis, via collision with a cell. This interaction exchanges some energy to the cell, and the photon itself will be deflected with a reduction in its energy. The energy absorbed by the cell will possibly create damage to the DNA leading to cell death. Photons are linear in direction, their course cannot be altered in the liver except by collision with tissue, therein lies the key disadvantage in treating hepatic tumors, as the normal tissues above and below a tumor will be in the path of the photon beam and receive similar radiation dose. The rate of energy loss as a function of depth in tissue is well known for every level of photon energy, with higher energy beams penetrating deeper into the body while giving up less energy in the first few centimeters of soft tissue. In the 1960s through the early 1980s, external beam radiation was actually delivery of photons from radioactive decay of ^{60}Cobalt. Although it yielded photon energies with sufficient penetrating power for most tumors, it could not be used for deep abdominal or pelvic tumors without delivering a much higher dose more superficially in normal tissues. In addition, the physical radiation beam itself had a relatively wide beam edge or penumbra, which made precise targeting impossible even at shallow depths of tissue. Over the past 20 years, linear accelerators have replaced ^{60}Cobalt machines virtually everywhere, and generate photons by accelerating electrons near to the speed of light before they strike a target, converting kinetic energy and mass into electromagnetic energy – photons. They generate photons of much higher energy than ^{60}Cobalt, and are thus able to reach any deep tumor in the body of most patients, without excessive "hot spots" or doses higher than that of the tumor along the photon path in

the body. In absolute numbers, ^{60}Cobalt can deliver gamma rays (photons) of two energies, 1.17 MeV (million electron volts) and 1.33 MeV, while some accelerators are capable of maximum photon energies of between 4 and 25 MeV, most centers use 6–18 MeV, which can easily safely reach the deepest parts of the liver in nearly any patient. Linear accelerators can also produce electron beams, which differ from photon beams, in that electrons are particles with mass and charge, and thus have a finite range of tissue penetrance, allowing for treatment of more superficial tumors, while significantly sparing deeper normal tissues. Electron beam therapy may be appropriate in treating a mass in the liver, which is only 1–2 cm deep to the surface. The dose 4 cm below the tumor could be nearly zero if the appropriate energy was chosen, compared to a dose of 80% of the tumor dose at that depth, if photons were used. Protons can be used similarly to electrons, but with a much deeper penetration if required (see later in chapter).

2.2. Radiation Dose

Dose of ionizing radiation absorbed by the liver, solid tumor, or other tissues is a cornerstone of clinical trial design. Older reports used the term roentgen (R), which described ionization in air, i.e., exposure of gamma rays. Newer nomenclature uses the SI unit for absorbed dose in tissue (1 J/kg = 1 gray (Gy) = 100 rads = 100 cGy (centigray)), as the basic unit of measurement. Conversion of older literature values listed as R is approximately 1 R = 0.01 Gy, for gamma. It is less well known how to convert beta radiation doses, which are low dose, constant release radiotherapy, into equivalent external beam doses due to the differences in biologic response due to dose rate, fractionation, and activity *(1)*. Thus brachytherapy doses are recorded as Gy, but these doses are not likely to be equivalent to the same dose Gy given as daily fractionated external beam doses of x-rays. This is an area of active investigation.

2.3. Three-Dimensional Conformal Radiation Therapy (3D-CRT)

Advances in software allow radiation oncologists to recreate volumetric models of patients using the latest and most detailed diagnostic images from CT or MRI. Typically CT data sets are used, and many cancer centers have dedicated spiral CT scanners in the radiation oncology department, hardwired to the treatment planning computer system. Two-dimensional treatment planning had been the only method prior to the mid-1990s, of planning how to arrange radiation beams targeting the tumor. This approach was limited to simple beam arrangements such as opposed beams, or those at 90° from each other (coplanar), and were designed from the standpoint of treating extra normal tissue so as to minimize the frequency of geometric miss of

the target by the beam. With precise targeting and tumor delineation as seen on CT volume sets, complex and innovative beam arrangements can be utilized with significant reduction in the need to include extra normal tissue as a margin. These noncoplanar beams can be at virtually any angle, although the linear accelerator and patient position will make some angles unusable. This approach also benefits from powerful new radiation dose calculations, which speed up the process of comparing alternate treatment plans by displaying nearly real-time dose maps. Enhancements also include the ability to more accurately calculate dose from beams that pass through less-dense tissues (inhomogeneity corrections), such as lung, in targeting the right lobe of liver *(2)*.

2.4. Fourth-Dimensional Conformal Radiation Therapy (4D-CRT)

The ability of real-time images taken during the delivery of radiation to a tumor (portal imaging or external imaging) has enabled further improvements in tumor targeting. Software algorithms that detect the tumor or fiducial markers placed near the tumor can control when the radiation beam is on or off. When treating a part of the body (i.e., lung or liver tumors) that changes position during respiration, the photon beam is interrupted when breathing causes the target to move out of the beam – termed "gaiting" or "respiratory gaiting." It does not depend upon rigid immobilization of the patient as in some forms of treatment.

2.5. Intensity-Modulated Radiotherapy (IMRT)

Intensity-modulated radiation therapy is a specialized application of 3D-CRT that allows radiation to be more exactly shaped to fit the tumor by varying the amount of radiation delivered to portions of the radiation field. The radiation beam can be subdivided into many "beamlets," and the intensity of each beamlet can be adjusted individually. Using IMRT, it has been possible to further limit the amount of radiation that is received by healthy tissue near the tumor. Most notably IMRT can spare salivary glands from permanent damage when treating head and neck malignancies, and reduce bladder and rectal complications in prostate cancer treatment. In some situations, this may also allow a higher dose of radiation to be delivered to the tumor, potentially increasing the chance of a cure.

2.6. Stereotactic Body Radiotherapy (SBRT)

Stereotactic radiotherapy is a technique of delivering fewer than normal fractions (hypofractionation) but each fraction is much larger than standard (2–3×). If given in a single dose it is considered "radiosurgery" which is reserved for CNS tumors and the skull is rigidly fixed to a frame. Liver

tumors are treated in 3–5 fractions with the body immobilized from chest to pelvis in specialized forms that are often custom fitted to the patient.

2.7. Image-Guided Radiation Therapy (IGRT)

IGRT involves conformal radiation treatment guided by imaging, such as CT, ultrasound, or X-rays, taken in the treatment room just before the patient is given the radiation treatment. All patients first undergo a CT scan as part of the planning process. The imaging information from the CT scan is then transmitted to a computer in the treatment room to allow a real-time comparison just before treatment to determine if the patient's position needs to be adjusted. This allows correction of patient positioning changes day to day, minute to minute, and any tumor changes over time.

2.8. Brachytherapy

It was not long after Dr. Wilhelm Conrad Roentgen discovered x-rays in 1895 that the *Lancet* reported its use in January 1896 for medical use *(3)*. Shortly after the turn of the century, it was suggested by Alexander Graham Bell that radioactive isotopes be applied directly to tissues, and thus *brachytherapy* was born – from the Greek "brachy" meaning "short range." The French coined the term endocurietherapy, Greek "endo" meaning "within." Radioactive isotopes such as iridium (^{192}Ir), cesium (^{137}Cs), and iodine (^{125}I and ^{131}I) have been used extensively since the early 1900s as primary therapy, and in addition to external beam radiation as a "boost" to the tumor. Brachytherapy attempts to spare normal regional tissues by delivering a high dose locally in the tumor, and although gamma radiation photons are used mostly, there is relatively low dose at a distance from the tumor of several centimeters. The dose rate of radiation delivery via a brachytherapy isotope (50 cGy/h) is much lower than photons delivered by an accelerator (100 Gy/min). Radioactive decay from an isotope that produces electrons (charge –1) is termed "beta decay." These particles are used in such products as radiolabeled antibodies used in hematologic malignancies, or in higher energies, for bone metastases and thyroid malignancies. Currently, there is significant clinical use of pure beta emitting isotopes (no gamma photons emitted) yttrium and strontium (^{90}Y, ^{90}Sr) in brachytherapy in liver lesions (see microsphere section) and in coronary artery brachytherapy. An advantage and potential disadvantage of beta sources is that most of the effective radiation is delivered within 2–4 mm of the source, with virtually no radiation dose effect >1 cm away. Because there are no gamma rays, nuclear medicine detectors cannot readily image pure beta sources, making localization of implanted sources problematic. Brachytherapy sources can be implanted via blood infusion, needle applicator, directly applied and sutured

into place as a permanent implant, or placed temporarily (minutes to hours) within a catheter that is removed from the body.

3. RADIOBIOLOGY

An understanding of radiation effects in living tissues began at the turn of the century with observations of skin reaction, primarily erythema and breakdown *(3)*. Since then clinical experience has produced observations regarding normal and malignant tissue response and repair to ionizing radiation. The target of efficient cell killing is the DNA, with the majority of cell death by irradiation resulting from unrepaired or misrepaired genomic injury, and loss of reproductive ability. It has been estimated that in the presence of sufficient oxygen tension (>10 mm Hg) *(3,4)* any form of radiation (x-rays, gamma rays, charged, or uncharged particles) will be absorbed and potentially interact directly or indirectly with the DNA. Approximately 75% of the damage to the DNA is *indirect*, with a photon striking a water molecule (water composes 80% of the cell) within 4 nm of the DNA strand. Kinetic energy from the incident photon is transferred to an orbital electron of the water molecule, ejecting it, now called a secondary electron. It can interact with a water molecule forming a free radical, which is highly reactive and breaks bonds in one of the DNA strands nearby. There can also be interaction of the secondary electron directly on the DNA strand causing damage, referred to as *direct* action *(3)*.

3.1. Modifiers of Radiation Response

The presence of oxygen is the single most important biologic modifier at the cellular/molecular level *(1,5)*. Oxygen "fixes" or makes permanent DNA damage caused by free radicals, but in low oxygen tensions, this damage can be repaired more readily. A term is used "oxygen enhancement ratio – OER" to describe the ratio of radiation doses without and with oxygen to produce the same biologic effect. For x-rays it is estimated to be between 2 and 3, i.e., a given x-ray will be 2–3 times as damaging in the presence of oxygen in that tissue than if hypoxia exists *(3)*. This has significant implications clinically as many HCC patients are considered for embolization procedures, which can produce a relative hypoxic environment within the tumor making them less susceptible to radiation therapy. Other factors can affect tumor sensitivity to radiation, including *repair* of radiation damage, *reassortment* of cells into more or less sensitive portions of the cell cycle (S phase most radioresistant, G2-M most sensitive), and *repopulation*, during a course of radiation, which are seen in rapidly dividing tumor populations. Repopulation can also become an issue after surgical resection, chemoembolization, cryotherapy, or radiofrequency ablation, where hepatic hypertrophy in the

regional normal cells is stimulated. These normal clonogens are more susceptible to radiotherapy damage in this phase, limiting the use of radiation, which may allow for residual malignant cells to repopulate *(6)*. Repair of radiation damage or "sublethal damage repair" is enhanced in low oxygen environments and with fractionation of radiation doses. The break between fractions in external beam radiotherapy provides opportunity to repair DNA strand breaks in normal and malignant cells. Brachytherapy differs in this regard with continuous radiation, without a discrete "fraction" of radiation, but it delivers continuous lower dose rate of radiation continually.

4. RADIATION EFFECTS IN THE LIVER

Acute and late effects of ionizing radiation to the liver have been described in the literature since the early 1960s *(7,8)*. During radiotherapy, acute or transient effects are often reported as elevation of liver enzymes, and depending upon the treated volume, hematologic effects such as neutropenia and coagulopathy can occur. However, permanent effects can be produced, occurring weeks or months after radiation (late effects) such as fibrosis, persistent enzyme elevation, ascites, jaundice, and rarely, radiation-induced liver disease (RILD) and fatal veno-occlusive disease (VOD) *(6, 9–11)*. RILD is often what is called "radiation hepatitis" and classically was described as occurring within 3 months of initiation of radiation, with rapid weight gain, increase in abdominal girth, liver enlargement, and occasionally, ascites or jaundice, with elevation in serum alkaline phosphatase. The clinical picture resembled Budd-Chiari syndrome, but most patients survived, although some died of this condition without proven tumor progression. It was described that the whole liver could not be treated with radiation above 30–35 Gy in conventional fractionation (1.8–2 Gy/day, 5 days per week) or else RILD or VOD was likely to occur. Interestingly, VOD can also occur without radiotherapy in patients receiving high-dose chemotherapy in hematologic malignancies, alkaloids, toxic exposure to urethane, asphenamine and long-term oral contraceptives, *(12)* as well as patients receiving radiation combined with chemotherapy or radiation alone. The clinical presentation can differ between RILD and chemotherapy+radiation liver disease, but the common pathological lesion associated with RILD is VOD. The pathologic changes in VOD can affect a fraction of a lobe or the entire liver. It is best observed on low power microscopy, which demonstrates severe congestion of the sinusoids in the central portion of the lobules with atrophy of the inner portion of the liver plates (zone 3) *(6,12)*. Foci of yellow necrosis may appear in the center of affected areas. If the affected area is large, it can produce shrinkage and a wrinkled granular capsule. The sublobular veins show significant obstruction by fine collagen fibers, which

do not form in the larger veins and (suprahepatic and cava) which is a distinction between RILD and Budd-Chiari syndrome *(6,12)*. Most livers heal and will display chronic changes after 6 months with little congestion, but distorted lobular architecture with variable distances between central veins and portal areas. These chronic liver changes are typically asymptomatic but are reproducibly seen on liver biopsies as late as 6 years after presentation. Further investigation of the pathogenesis of VOD is difficult as most animals do not develop VOD in response to radiation *(12)*.

5. CLINICAL STUDIES

5.1. External Beam Radiation Therapy (EBRT)

Because of the tolerance issues of normal liver to radiation as discussed earlier, there has been little activity regarding radiation alone for HCC. With improvements in targeting with 3D-CRT, however, there is renewed interest in combining radiation with chemotherapy and other modalities. Most radiation oncologists use external beam radiation in the liver for palliation of symptoms such as pain secondary to capsular stretching from tumor expansion or intratumoral hemorrhage. Definitive therapy attempts in unresectable HCC using radiation have only recently been published with the appearance of toxicity data from carefully done clinical studies using CT-based 3D-CRT. Seminal work by Lawrence and colleagues at the University of Michigan over the past decade has significantly increased our understanding of liver tolerance to radiotherapy and combined chemoradiotherapy *(6,10,11, 13–22)*. With extensive clinical experience using 3D-CRT in daily and twice daily radiation fractions, and combined with hepatic artery infusion of different chemotherapy agents, a clearer understanding now exists as to the limits of this approach, and predictive models of RILD created to design the next generation of clinical trials *(10,23–25)*.

Mornex *(26)* reported a phase II trial of 27 patients that included both Child-Pugh A and B cirrhotic patients with small-size HCC (1 nodule $\leq$5 cm or 2 nodules $\leq$3 cm) who are not candidates for curative treatments. High-dose (66 Gy, 2 Gy/fraction) 3D-CRT was used for all patients. In the 25 assessable patients, tumor response was observed for 23 patients (92%), with complete response for 20 patients (80%), and partial response for 3 patients (12%). Stable disease was observed in 2 patients (8%). Grade 4 toxicities occurred in 2 of 11 (22%) Child-Pugh B patients only. Child-Pugh A patients tolerated treatment well and 3/16 (19%) developed asymptomatic grade 3 toxicities *(26)*.

Predictive models of normal tissue complication probability (NTCP) use clinical outcomes from partial liver radiotherapy and chemoradiotherapy experiences, based on quantified volumes of the liver that received a specific

dose of radiation, which lead to RILD or other toxicity. They incorporate the entire treatment plan, and can describe dose–volume relationships of the liver between inhomogeneous dose distributions *(10)*. Dose escalation trials reported by Dawson have shown safety and tumor regression in HCC and other hepatobiliary cancers with doses between 28.6 and 90 Gy in combination with concurrent hepatic artery infusion of fluorodeoxyuridine *(19)*. A response rate of 68% was achieved, with only 1 case of RILD, grade 3, which was reversible, and no treatment-related deaths. The team saw, not surprisingly, a dose–response advantage in progression-free survival for the 70–90 Gy cohorts. No MTD has been reached, and radiation dose escalation is ongoing *(19)*.

Multicenter cooperative group trials have only been attempted by the Radiotherapy Oncology Group (RTOG) which pre-dated 3D-CRT and NTCP modeling, which now enable partial liver doses of >90 Gy. The first, RTOG 83-19, tested the addition of ^{131}I-antiferritin monoclonal antibodies to doxorubicin plus 5-fluorouracil to patients that first had entire liver radiotherapy to 21 Gy in large daily fractions of 3 Gy *(27)*. This study is very different in design to current liver radiotherapy practice, which uses smaller fractions bid or daily, partial liver volumes, and hepatic artery infusion chemotherapy and/or TACE (transarterial chemoembolization). Single fraction doses above 2 Gy per day are known to increase late effects in the end organ, such as fibrosis, whereas small fractions given twice daily are believed to spare the organ from late injury, i.e., RILD *(3)*. The outcome of the RTOG experience was negative with ^{131}I-antiferritin, and the successor trial (RTOG 88–23) was also negative, with the same radiotherapy components, but a chemotherapy change using cisplatin, which suggested some activity to the combination *(28)*.

5.1.1. External Beam Radiation (3D-CRT/IMRT) and TACE

External beam radiation for unresectable HCC, in total doses greater than 35 Gy with TACE, for salvage of initial TACE failures *(29–31)*. Seong reported the use of 3D-CRT (mean tumor dose 44 ± 9.3 Gy) in combination with chemoembolization with doxorubicin and lipiodol in 30 patients with unresectable HCC. In this small group, a 63.3% objective response was noted and median survival of 17 months without a treatment-related death *(29)*. In a subsequent report, Seong delivered (mean tumor dose 51.8 + 7.9 Gy) external beam radiation to 24 patients with unresectable HCC who had progressed after TACE with lipiodol–adriamycin mixture. He noted an encouraging response rate of 66.7%, 3-year survival rate of 21.4%, and no treatment-related deaths *(30)*. In an update on both previously reported groups and additional patients treated to a total of 158 (107 patients concurrent with TACE, 51 as salvage), Seong analyzed prognostic factors for response rate and overall survival. On univariate analysis tumor

size, portal vein thrombosis, and radiation dose were significant, but only radiation dose was significant on multivariate analysis. The mean radiation dose to the tumor for the entire cohort was 48.2 ± 7.9 Gy at 1.8 Gy/day *(31)*. Park studied the same patient cohort as Seong, and determined a dose–response relationship existed, with dose groupings of <40, 40–50, and >50 Gy *(30,31)*. An autopsy study of 7 patients after radiotherapy for HCC suggested viable tumor remained despite doses of 50–70 Gy *(32)*. Using 2D treatment planning to deliver external beam x-rays with TACE, Guo reported the result in 107 patients with unresectable HCC. This retrospective study also found increasing radiation dose to be a prominent factor in objective tumor response, as well as number of tumors. The radiation dose range was 22–55 Gy in 1.6–2.0 Gy/day fractionation using moving strip technique to treat the entire liver in 78 patients *(33)*.

Guo *(34)* reported a comparison of 76 patients with large unresectable HCC treated with TACE followed by external beam irradiation and a control group of 89 patients with large HCC, who underwent TACE alone during the same period. Clinical features, therapeutic modalities, acute effects, and survival rates were analyzed and compared between TACE plus irradiation group and TACE alone group. Multivariate analysis of nine clinical variables and one treatment variable (irradiation) was performed by the Cox proportional hazards model. The clinical features and therapeutic modalities except irradiation between the two groups were comparable ($P>0.05$). The objective response rate (RR) in TACE plus irradiation group was higher than that in TACE alone group (47.4% versus 28.1%, $P<0.05$). The overall survival rates in TACE plus irradiation group (64.0, 28.6, and 19.3% at 1, 3, and 5 years, respectively) were significantly higher than those in TACE alone group (39.9, 9.5, and 7.2%, respectively, $P=0.0001$). Cox proportional hazards model analysis showed that tumor extension and Child grade were significant and were independent negative predictors of survival, while irradiation was an independent positive predictor of survival. The authors concluded that TACE combined with radiotherapy is more effective than TACE alone, and is a promising treatment for unresectable large HCC.

Zeng *(35)* retrospectively studied 203 patients that received TACE for unresectable HCC. None had tumor thrombus, lymph node involvement, or extrahepatic metastasis based on computed tomography scans of the chest and abdomen. Among these 54 also received combination therapy with external beam radiotherapy. Tumor response rate, survival, and failure patterns were analyzed and compared between the two groups. Objective responses (complete and partial responses) on computed tomography study were 31 and 76% without radiotherapy and with radiotherapy, respectively. Overall survival rates in the radiotherapy group were 71.5, 42.3, and 24.0% at 1, 2, and 3 years, respectively, improved over the non-radiotherapy group rates of 59.6, 26.5, and 11.1% at 1, 2, and 3 years, respectively. Intrahepatic

failure was lower in the radiotherapy group than in the non-radiotherapy group, but the difference was not significant. Side effects from radiotherapy were common, but rarely severe.

5.1.2. External Beam Monotherapy

Kim *(36)* used 3D-CRT for unresectable HCC patients for whom TACE was ineffective or unsuitable, and to determine whether tumor response and portal vein thrombosis (PVT) response to treatment were prognostic factors for overall survival. From July 2001 to June 2005, 70 unresectable HCC patients were treated; PVT was present in 41 patients. Fraction size was 2–3 Gy daily via x-rays to a total dose of 44–54 Gy. Follow-up CT evaluations showed primary tumor responses: complete response in 4 (5.7%) patients, partial response in 34 (48.6%), no response in 28 (37.1%), and progressive disease in 4 (8.6%). Of 41 patients with PVT, the PVT responses were CR in 4 (9.7%) patients, PR in 12 (29.3%), NR in 20 (48.8%), and PD in 5 (12.2%). The median survival times were 18.0 and 20.1 months in the primary tumor and the PVT responders (CR + PR), respectively, which were longer than 6.8 and 7.2 months in the primary tumor and the PVT nonresponders (NR + PD), respectively. An overall 54.3% objective response rate for primary tumors and a 39.0% objective response rate for PVT were seen. Both primary tumor and PVT responses were prognostic factors for overall survival. The authors concluded that 3D-CRT is a practical treatment option in HCC patients for whom TACE is ineffective or unsuitable.

Liu *(37)* also used 3D-CRT for patients who had either failed with or were unsuited for TACE. A total of 44 patients with unresectable HCC underwent 3D-CRT, mean age was 62 years ranging from 34 to 88. Eastern Cooperative Oncology Group (ECOG) performance status was 0 in 10 patients, 1 in 19 patients, and 2 in 15 patients. Child-Pugh classification was A in 32 patients and 12 patients in class B with 14 patients having main portal vein thrombosis. Tumor size was <5 cm in 16 patients, 5–10 cm in 16 patients, and >10 cm in 12 patients. Thirty-two patients had tumors of confluent type, the remaining patients presented a single hepatic tumor. An objective response was observed in 27 of 44 patients, giving a response rate of 61.4%. The survival rates at 1, 2, and 3 years were 60.5, 40.3, and 32.0%, respectively. A significant impact on survival was found for several factors including total dose of radiotherapy.

5.1.3. External Beam Radiotherapy for Portal Vein Thrombosis

Several investigators have used 3D-CRT and stereotactic radiotherapy successfully to treat the portal vein tumor and not the primary HCC lesions. Overall the response rate is approximately 80% with very few side effects.

5.1.4. Proton (External Beam) Radiotherapy

Proton radiation therapy (simply referred to as "protons") has been used with success for HCC with most published data from Japan. A fundamental difference between x-rays of traditional external beam radiotherapy and protons is that because protons carry a charge and have mass (photons are electromagnetic waves, no charge or mass), protons can be delivered into deep tissues with lower radiation deposition above and below the target compared to x-rays, releasing nearly all of their energy within the tumor. Because of the enormous cost of constructing these accelerators ($100 million USD per facility), which require a cyclotron onsite, they are only currently available at four centers in the United States and several other centers worldwide. Clinical use of protons is mostly for CNS, spinal cord, ocular, base of skull, head and neck, and prostate tumors. Protons have similar efficacy to x-rays in destroying tumor cells, but more normal tissue can be spared due to its physical dose deposition characteristics *(38)*. Between 1983 and 2000, the Proton Medical Research Center at the University of Tsukuba treated more than 236 patients with HCC. The dose/fraction was 4.5 Gy daily to a total dose of 72 CGE in 3.2 weeks. Dose is quoted in CGE to denote the dose in Gy multiplied by the radiation biologic effectiveness unit, 1.10 (x-rays are 1.0). For small HCC tumors, Tokuuye reported a 3-year actuarial local control rate of 93% *(39)*. Matsuzaki reported the use of protons for 24 patients failing TACE for HCC and found tumor response in >90% of these lesions *(40)*. Proton beam therapy may become more common as new facilities that are currently planned worldwide become operational.

5.1.5. Stereotactic Body Radiotherapy (SBRT) Studies

Stereotactic radiotherapy has been studied in a phase I/II trial of mixed neoplasia in the liver, which included one HCC patient. Herfarth demonstrated feasibility of the technique to deliver 14–26 Gy in a single fraction to the liver (with the 80% isodose surrounding the planning target volume) to 60 tumors in 37 patients *(41)*.

Wu *(42)* used SBRT combined with TACE in 94 patients with cirrhosis and HCC. A total of 63 patients had Okuda stage I lesion and 31 patients had stage II. The median tumor size was 10.7 cm (range 3.0–18 cm). There were 43 cases of class A and 51 cases of class B. TACE contained lipiodol, 5-fluorouracil, cisplatin, doxorubicin hydrochloride, and mitomycin, followed by gelatin sponge cubes. Fifty-nine patients received a single TACE delivery while the remaining patients received two or three TACE procedures. Radiotherapy began 3–4 weeks after the last TACE procedure. All patients were irradiated with a stereotactic body frame and received 4–8 Gy single high-dose radiation for 8–12 times at the isocenter during a period of 17–26 days (median 22 days). The median follow-up was 37 months (range

10–48 months) after diagnosis. The response rate was 90.5% and overall survival rate at 1 year, 2 years, and 3 years was 93.6, 53.8, and 26.0%, respectively, with the median survival of 25 months. In univariate and multivariate analyses, age, tumor size, and radiation dose (P=0.001) were significant prognostic factors for survival.

Tse *(43)* completed a phase I study of individualized SBRT for unresectable HCC and intrahepatic cholangiocarcinoma (IHC) not suitable for standard therapies. Six fractions of SBRT over 2 weeks were delivered with total radiation dose dependent on the volume of liver irradiated and the estimated risk of liver toxicity based on a normal tissue complication model (NTCP). Toxicity risk was escalated from 5 to 10 and 20%, within three liver volume-irradiated strata, provided at least three patients were without toxicity at 3 months after SBRT. Forty-one patients with unresectable Child-Pugh A HCC ($n = 31$) or IHC ($n = 10$) completed six-fraction SBRT. Five patients (12%) had grade 3 liver enzymes at baseline. The median tumor size was 173 mL (9–1,913 mL). The median dose was 36.0 Gy (24.0–54.0 Gy). No radiation-induced liver disease or treatment-related grade 4/5 toxicity was seen within 3 months after SBRT. Seven patients (5 HCC, 2 IHC) deteriorated in liver function from Child-Pugh class A to B within 3 months after SBRT. Median survival of HCC and IHC patients was 11.7 months (95% CI, 9.2–21.6 months) and 15.0 months (95% CI, 6.5–29.0 months), respectively.

5.2. Brachytherapy

5.2.1. ^{131}I-Lipiodol

Most commonly, brachytherapy for HCC has been accomplished by hepatic artery infusion of ^{90}Y-embedded microspheres or ^{131}I-lipiodol. The rationale for hepatic artery infusion is anatomic observation that tumors receive >80% of their blood supply from the hepatic artery, as opposed to normal hepatic triads, which receive the converse 80% supply of nutrients from the portal system. With the tumor/normal tissue ratio thus favorable from the hepatic artery, lipiodol, used for years in non-radiation embolic therapy in the liver (containing 38% iodine by weight), was a logical choice to add a radioisotope. In animal studies, ^{131}I-lipiodol had a significantly longer half-life in tumor as opposed to normal liver parenchyma. ^{131}I is a pure beta emitter with limited range penetration of electrons, thereby sparing normal liver adjacent to the tumor from significant dose. In an excellent review of clinical studies using ^{131}I-lipiodol by Ho, there were 14 studies between 1985 and 1997, with more than 400 patients having received this therapy *(44,45)*. Most patients were treated with unresectable HCC for amelioration of symptoms; response rates were 25–70% in uncontrolled studies.

Raoul reported a multicenter randomized study of patients with portal vein thrombosis from HCC who received 10–100 Gy in 1–5 injections and had better survival than the control (untreated) group *(45)*. In a separate prospective trial of 142 patients with unresectable HCC, randomization was to ^{131}I-lipiodol versus chemoembolization with cisplatin (70 mg). There was no difference in survival or tumor response between the two therapies; however, toxicity was less with ^{131}I-lipiodol *(46)*.

In the adjuvant setting, postoperative ^{131}I-lipiodol has been tested in a prospective randomized trial by Lau that was stopped early. Randomized patients after resection in the experimental arm received ^{131}I-lipiodol (1,850 MBq in a single dose) or no further therapy (control group). Interim analysis of 21 treated and 22 control patients showed a statistically significant decrease in recurrence (28.5% versus 59%), and improved median disease free survival (57.2 months versus 13.6 months) for the treated patients *(47)*.

Lau *(47)* updated long-term results from a prospective randomized trial of postoperative adjuvant intra-arterial iodine-131-labeled lipiodol in HCC. Early results after closing the trial showed that 1 dose of intra-arterial ^{131}I-lipiodol given after curative resection significantly decreased the rate of recurrence, and increased disease-free and overall survival. Patients who underwent curative resection for HCC and recovered within 6 weeks were randomly assigned one 1,850 MBq dose of I-lipiodol or no further treatment (controls). Comparison of rates of recurrence and long-term disease-free and overall survival (the primary endpoints) between the 2 groups by intention-to-treat was completed on 43 patients in total (21 in radiation group, 22 controls). I-lipiodol had no significant toxic effects. During a median follow-up of 66 (range 3–198) months, there were 10 (47.6%) recurrences among the 21 patients in the adjuvant treatment group, compared with 14 (63.6%) in the control group ($P = 0.29$). The actuarial 5-year disease-free survival in the treatment and control groups was 61.9 and 31.8%, respectively ($P = 0.0397$). The actuarial 5-year overall survival in the treatment and control groups was 66.7 and 36.4%, respectively ($P = 0.0433$). The actuarial 7-year disease-free survival in the treatment and control groups was 52.4 and 31.8%, respectively ($P = 0.0224$). The actuarial 7-year overall survival in the treatment and control groups was 66.7 and 31.8%, respectively ($P = 0.0243$). The actuarial 10-year disease-free survival in the treatment and control groups was 47.6 and 27.3%, respectively ($P = 0.0892$). The actuarial 10-year overall survival in the treatment and control groups was 52.4 and 27.3%, respectively ($P = 0.0905$). The authors concluded that adjuvant intra-arterial I-lipiodol after curative liver resection provided a survival benefit – disease-free survival and overall survival, although the difference became statistically insignificant at 8 years after randomization.

5.2.2. ^{90}Y-Microspheres (Yttrium-90)

The rationale for microsphere treatment is infusion of a sphere charged with ^{90}Y which will undergo beta decay with energetic electrons penetrating only 2–8 mm, over a half-life of 64 h. Microspheres range in diameter of between 20 and 40 μm such that they will become embedded within the tumor vasculature, but because the end arterioles are <10 μm in diameter, they will not pass into the venous circulation. The lungs are the next arteriole bed, which would capture the spheres (Figs. 1 and 2). Pulmonary tolerance to radiation is roughly half (< 20 Gy) that of the liver and unintentional deposition of microspheres with ^{90}Y has led to deaths in past trials *(48,49)*. Arteriovenous shunts in the liver that would allow free passage of microspheres into the venous system and then to the lungs are not readily apparent on angiogram. Therefore, patient screening involves detailed hepatic angiographic mapping coupled with a nuclear imaging, using albumin tagged with a gamma emitter technetium-99 (^{99m}Tc-MAA) injected into the hepatic artery. It is then possible to calculate the percentage of shunting of ^{99m}Tc in the lung compared with the known amount infused into the liver. Typically if >10–15% of the dose appears in the lungs, a dose reduction of

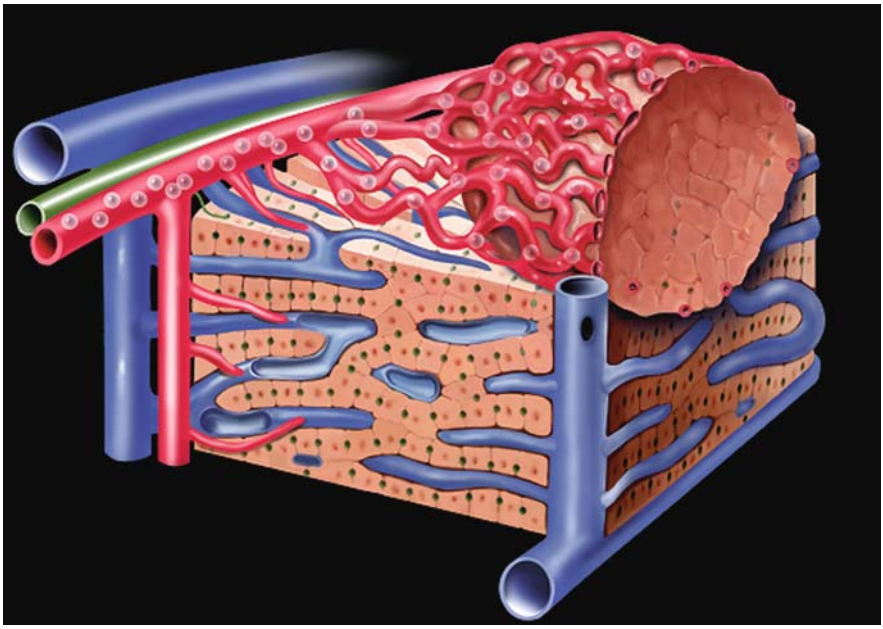

Fig. 1. Illustration of the arterial plexus of abnormal vessels recruited by hepatocellular cancers and the route ^{90}Y microspheres take to embed into the tumor. The beta radiation emitted only penetrates 3–4 mm from each microsphere sparing the adjacent normal liver tissue beyond the tumor.

Fig. 2. A full dose of ^{90}Y microspheres about to be delivered intra-arterially via the hepatic artery. A small volume (2 cc) of microspheres is resting at the bottom of a vial, with the vial contained in an acrylic case to protect the staff from receiving radiation exposure.

microspheres is attempted or the procedure is aborted *(50–52)*. Infusion of the entire liver can be accomplished in a single infusion, however, this will increase toxicity versus a sequential lobar approach, with a 4-week interval between infusions *(50)*.

A consensus panel *(53)* provided category 2a consensus evidence and guidelines for employing internal liver radiotherapy with radioactive microspheres. Among its purposes was to standardize the indications, techniques, multimodality treatment approaches, and dosimetry to be used for yttrium-90 microsphere hepatic brachytherapy. Members of the Radioembolization Brachytherapy Oncology Consortium (REBOC) were independent group of experts in interventional radiology, radiation oncology, nuclear medicine, medical oncology, and surgical oncology that identified areas of consensus and controversy and issued clinical guidelines for ^{90}Y microsphere brachytherapy. A total of 14 recommendations were made with key findings including sufficient evidence that exists to support the safety and effectiveness of ^{90}Y microsphere therapy. A meticulous angiographic technique is required to prevent complications. Resin microsphere prescribed activity is best estimated by the body surface area method. By virtue of their training, certification, and contribution to ^{90}Y-microsphere treatment programs, the disciplines of radiation oncology, nuclear medicine, and interventional radiology are all qualified to use ^{90}Y-microspheres. The panel strongly advocated the creation of a treatment registry with uniform reporting criteria.

Initiation of clinical trials to further define the safety and role of ^{90}Y-microsphere in the context of currently available therapies is needed. Also included was a summary of HCC trials of ^{90}Y-microspheres which showed a favorable toxicity profile, response rate, and overall survival in a difficult group of patients.

Ariel and Simon were the first investigators to perform microsphere clinical trials in humans. Most patients had metastatic carcinoid or colorectal cancers in the early 1960s *(54–56)*. Their pioneering work was with composite spheres and ^{90}Y but their treatment procedures for screening, infusion, and post treatment imaging are largely intact in modern clinical practice *(50,57–66)*. There are two microsphere devices available in the United States, the glass microsphere (TheraSphere®) and resin-based sphere (SIR-Spheres®), which are similar in size and isotope (^{90}Y) but have some important differences in delivery and physical characteristics *(67)* (Table 1). Both began in clinical trials in the late 1980s and have been used in thousands of

Table 1
Comparison of Radioactive Microsphere Agents

Parameter	*Glass*	*Resin*
Size (median)	25 μm	32 μm
Isotope	^{90}Y	^{90}Y
Number of spheres in standard dose	4 million (range 2–8 million)	40 million (range 30–80 million)
Total activity infused in typical treatment	5 GBq (range 3–20 GBq)	1.8 GBq (range 0.8–3.0 GBq)
Activity per microsphere for typical treatment	2,500 Bq	50 Bq
Indication(s)	HCC (USA) HCC & Colon (Canada)	Colon (USA) All tumor types (Europe, Asia)
Regulatory status (United States FDA)	Humanitarian device exemption (HDE) HCC only	Pre-market approval (PMA) colorectal cancer liver metastases
Limitations on treatment	High radiation dose in cirrhotic patients	High risk of embolic complications due to large number of microspheres

patients since, mostly with colorectal metastases, but sufficient HCC patients have been treated to make some observations *(48,51,58,61,62,68–82)*.

Carr presented a report of a phase II trial of glass microspheres via lobar approach, with a nominal target dose of 135 Gy *(71,74)* and a quality of life companion study *(72,83)*. He also statistically compared survival of published untreated Okuda I and II patients *(84–86)* to his study cohort *(72,74)*. Tumor reductions were documented in 42 patients (64.6%) via decreased vascularity, with 25 patients (38.4%) having a partial response by CT. Median survival for Okuda stage 1 (42 patients) was 649 days (360–1,012) compared to historical median of 244 days. The advantage was even more pronounced in those with Okuda stage II (23 patients) with a median survival after microspheres of 302 days (166–621) versus a historical median survival of 64 days. Toxicity and quality of life were good, with only 1 patient judged to have died related to microsphere therapy. The quality of life report of this patient group compared hepatic artery infusion with cisplatin versus microspheres, revealing a small advantage to microsphere therapy. Toxicity and survival in a group of 14 patients with unresectable HCC by Kennedy *(87)* and 16 patients by Soulen *(88)* were very similar to those reported by Carr, with elevated enzymes, nausea, and fatigue the most frequent common toxicity grade 2 or 3 findings. The dose delivered was different in all three studies: Kennedy *(87)* delivered a median dose of 149 Gy (128–174) to the whole liver with a 9-month survival of 75%, Soulen *(88)* a mean of 128 Gy (97–182), and Carr at 133 Gy *(72)*.

5.2.3. Additional Phase I–II ^{90}Y-Microsphere Trials in HCC

Lau *(69)* reported a phase I study of resin microspheres in 18 patients with inoperable HCC via an arterial port placed during laparotomy. The radiation doses to the liver and tumor were determined intraoperatively with a beta probe and liquid scintillation counting of multiple liver biopsies. The treatment was well tolerated without major complications. Response by tumor marker occurred in all patients and ranged from 41 to 0.2% of the pretreatment level. Tumor regression was correlated with radiation dose. Progressive or static disease occurred in a higher proportion of patients whose tumors received < 120 Gy ($P = 0.005$). Survival was improved if tumors received $>$ 120 Gy (median survival $= 55.9$ weeks) compared to lower doses (median survival $= 26.2$ weeks) which was significant ($P = 0.005$).

Lau *(68)* reported a phase II study involving 71 patients with HCC that had not had prior TACE or radiation therapy. Microspheres were infused into the hepatic artery at the time of hepatic angiography or through an implanted arterial portacatheter under fluoroscopy. Repeated treatments were given for residual or recurrent tumor. Response to treatment was monitored by serum alpha-fetoprotein or ferritin levels, together with serial CT scans. Of the 71

patients, 20 patients were treated for postoperative recurrence. Activity of ^{90}Y for the first treatment ranged from 0.8 to 5.0 GBq (21.6–135.1 mCi) with a median of 3.0 GBq (81.1 mCi). There was a 50% reduction in tumor volume in 19 (26.7%) patients after the first treatment. However, the overall objective response in alpha-fetoprotein levels was 89% (PR 67% plus CR 22%) among the 46 patients with elevated pretreatment levels. The serum ferritin level in the other 25 patients dropped by 34–99% after treatment. Treatment was repeated in 15 patients with the maximum number of treatments in an individual patient of 5 and the maximum total activity delivered in a single patient was 13.0 GBq (351.4 mCi) over 3 treatments. The estimated radiation doses to normal liver ranged from 25 to 136 Gy (median 52 Gy) in the first treatment and the highest total radiation dose was estimated to be 324 Gy. Tumor doses were 83–748 Gy (median 225 Gy) in first treatments and the highest cumulative dose reached was 1,580 Gy. The residual tumors were resected in 4 patients and in 2 of these patients no residual tumor was found and in the remaining 2 patients only occasional viable tumor cells were found in the necrotic centers of the tumors. The median survival of the 71 patients was 9.4 months (range 1.8–46.4 months). Treatment was well tolerated without serious adverse events, RILD or radiation pneumonitis.

Dancey *(48)* reported a phase II trial of glass microspheres for unresectable HCC of 22 patients, with only 20 receiving treatment. The median age was 62.5 years and overall performance status was Eastern Cooperative Oncology Group (ECOG) 0–3. A planned dose of 100 Gy was delivered through a femoral catheter approach to the hepatic artery. Nine patients were Okuda stage I and 11 were Okuda stage II. The median dose delivered was 104 Gy (range 46–145 Gy). All treated patients experienced at least one adverse event. Of the 31 (15%) serious adverse events, the most common were elevations in liver enzymes and bilirubin and upper GI ulceration. The response rate was 20%. The median duration of response was 127 weeks; the median survival was 54 weeks. Multivariable analysis suggested that a dose greater than 104 Gy ($P = 0.06$), tumor-to-liver activity uptake ratio greater than 2 ($P = 0.06$), and Okuda stage I ($P = 0.07$) were associated with longer survival. The authors concluded that significantly higher doses of radiation can be delivered to a HCC tumor by intrahepatic arterial administration of ^{90}Y-microspheres than by external beam radiation, although they did not test external beam radiation in their study *(48)*.

Kulik *(89)* and co-investigators reported results of a phase II trial of glass microspheres completed at two centers involving 108 patients with unresectable HCC with and without portal vein thrombosis. Patients treated were stratified by Okuda, Child-Pugh, baseline bilirubin, tumor burden, Eastern Cooperative Oncology Group (ECOG), presence of cirrhosis, and portal vein thrombosis (PVT) (none, branch, and main). Clinical and biochemical data were obtained at baseline and at 4-week intervals following treatment

up to 6 months. Tumor response was judged from CT scans. Thirty-seven (34%) patients had PVT, 12 (32%) of which involved the main PV. The cumulative radiation dose for those with and without PVT was 139.7 and 131.9 Gy, respectively. Radiographic response using WHO criteria was partial in 42.2%. Using European Association for the Study of the Liver (EASL), the response rate was 70%. The adverse event (AE) rates were highest in patients with main PVT and cirrhosis. There were no cases of radiation pneumonitis. Kaplan–Meier survival varied depending on location of PVT and presence of cirrhosis; with no PVT group median survival of 15.6 months (P=0.0052) was superior compared to all other patients. The best survival was in the non-cirrhotic, non-PVT patients with a median survival of 27.1 months (P=0.027) versus all others.

Estimating dose delivered in the tumor versus normal liver is problematic in microsphere therapy *(90–94)*, but it is clear from the literature that for the doses commonly used today and reported in either glass or resin spheres, the toxicity profile is fairly low and responses by imaging and tumor markers consistently good and in agreement between various researchers. With the widespread availability of this modality in Europe, North America, and Asia, increasing numbers of centers are beginning treatment protocols using microspheres alone or in combination with chemotherapy.

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24 Psychosocial Issues in Hepatocellular Carcinoma

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CONTENTS

ABSTRACT

The evaluation and treatment of psychosocial problems in patients diagnosed with HCC is critical as unmet psychosocial needs or distress can increase morbidity and mortality in patients diagnosed with HCC. This chapter will provide clinicians with (1) information regarding modifiable risk factors in the development of HCC; (2) tools to facilitate the evaluation of psychosocial distress and cancer-related symptoms in HCC; (3) a brief introduction of the emotional responses commonly expressed in

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patients with HCC; (4) frequently presenting psychological disorders in patients diagnosed with HCC; (5) common cancer-related symptoms in which behavioral treatments that can be employed to complement conventional pharmacological treatment; and (6) information regarding issues related to caregiving, cultural and religious factors in the treatment of HCC, end of life issues, and alternative and complementary medicine in the treatment.

Key Words: Psychosocial issues; depression; evaluation and treatment of psychiatric symptoms; psychiatric distress

1. PSYCHOSOCIAL EVALUATION AND TREATMENT OF DISTRESS IN ONCOLOGY

The National Comprehensive Cancer Network (NCCN) has deemed "psychosocial distress" as the sixth vital sign and has recently developed guidelines for management of distress in people diagnosed with cancer and their families *(1)*. According to the National Cancer Institute (NCI), distress may be defined as "extreme mental or physical pain or suffering." *(2)* Evidence continues to accumulate regarding the prevalence of psychological distress in patients diagnosed with cancer. Zabora and colleagues (2003) found in a sample of over 4,000 cancer patients that 25–43% reported significant distress *(3)*. Liver cancer was reported to have the third highest level of distress *(3)*. Due to the current pressures within the healthcare system, distress often goes unrecognized by healthcare providers. Fallowfield and colleagues found that only 29% of oncology patients who exceeded the cutoff score on a distress instrument were identified by their physicians as being distressed *(4)*. As a result of the increasing recognition of distress in people diagnosed with cancer, the Institute of Medicine (IOM) released a report that recommended the comprehensive screening, evaluation, and treatment of psychosocial needs of cancer patients and their families *(5)*. Academic and community oncology practices across the country are now implementing programs to begin to meet these recommendations.

The objective of this chapter will be to provide a framework to facilitate the goals of the IOM recommendations in patients diagnosed with hepatocellular carcinoma (HCC). The chapter will provide clinicians with (1) information regarding modifiable risk factors in the development of HCC; (2) tools to facilitate the evaluation of psychosocial distress and cancer-related symptoms in HCC; (3) a brief introduction of the emotional responses commonly expressed in patients with HCC; (4) frequently presenting psychological disorders in patients diagnosed with HCC; (5) common cancer-related symptoms in which behavioral treatments that can be employed to complement conventional pharmacological treatment;

and (6) special issues associated with HCC including caregiving, caring for children and adolescents, cultural and religious factors in the treatment of HCC, end of life issues, and alternative and complementary medicine. Due to the paucity of psychosocial research that has been conducted in HCC, the research that will be presented in this chapter will rely primarily on previous research with other cancer populations as well as research conducted by our team with patients and their families who are affected by hepatocellular carcinoma. The evaluation and treatment of psychosocial problems is critical as unmet psychosocial needs or distress can increase morbidity and mortality in patients diagnosed with HCC *(6*, *7)*.

2. THE ROLE OF BEHAVIOR IN THE DEVELOPMENT OF HCC

Hepatocellular carcinoma is the sixth most common cancer in the world *(8)*. Increasing evidence suggests that the development of cancer is likely a result of an interaction between genes, environment, and/or behavior *(9–16)*. As with HCC, not all individuals with known risk factors develop HCC. At this time genetic predisposition of HCC is not likely to be modified, however, the behavioral or environment risk factors associated with HCC may be prevented or modified to reduce the risk of this cancer.

The primary risk factors of HCC include hepatitis B and C (HBV and HCV); alcohol-related liver disease (ALD); nonalcoholic steatohepatitis (NASH; which is often associated with obesity, type II diabetes, dyslipidemia, and insulin resistance); and to a lesser extent congenital diseases such as hemochromatosis, alpha-l-antitrypsin deficiency, glycogen storage disease, porphyria cutanea tarda, tyrosinemia, aflatoxins, and Wilson disease; and in rare cases, biliary cirrhosis *(8*, *17–25)*. The majority of cases of HCC worldwide and in North America are secondary to HBV and HCV infection followed by NASH and alcohol abuse/dependence, which are modifiable risk factors. Increasing evidence suggests that in the next decade NASH will be the primary risk factor for HCC in North America and Europe *(26*, *27)*.

Some factors that contribute to the development of HCC (e.g., substance abuse/dependence) may also contribute to more rapid disease progression and medical complications *(28–30)* once diagnosed with HCC. Primary prevention in the form of education regarding risk factors and modes of transmission and interventions to reduce the incidence of risk behavior (e.g., substance abuse/dependence) may be instituted to reduce the risk of developing HCC. Nonalcoholic steatohepatitis is increasing in incidence and is expected to be the leading cause of HCC in North America *(26*, *27)*. In the last decade, the rate of obesity has doubled in adults and tripled in children *(31)*. Increased body mass index (BMI) leads to hyperlipidemia, hypertension, and diabetes *(32–39)*. Prevention through the improvement of

health behaviors (increased fruits and vegetables and physical activity) as well as the treatment of hyperlipidemia and diabetes may reduce the risk of NASH-related HCC.

Although less studied, environmental and/or occupational exposure and substances such as tobacco may play a role or have a synergistic effect in the development of HCC *(40–49)*. Tobacco use has been demonstrated to be associated with increased risk of cancer *(50–52)*. Alcohol and tobacco, along with infection with HCV, have been found to have a synergistic effect in the development of HCC *(41*, *44*, *46*, *47*, *53)*. In two studies, the combination of HCV with alcohol and/or tobacco was found to increase the risk for the development of HCC 5.6–7.2 times when compared to cirrhotic patients without these risk factors *(41*, *46*, *53*, *54)*.

The most common environmental risk factor is the exposure to aflatoxins. Aflatoxins, a mycotoxin formed by certain *Aspergillus* species, are a frequent contaminant of improperly stored grains and nuts. In parts of Africa, the high incidence of HCC in humans may be related to ingestion of foods contaminated with aflatoxins *(55–57)*. Limited evidence is available for other environmental risk factors; however, research suggests that both arsenic *(58*, *59)* and radiation exposure *(60)* may be associated with the development of HCC. Prevention programs to reduce exposure to HBV, HCV, and environmental/occupational hazards as well as programs to reduce tobacco and/or alcohol dependence are critical in the prevention of this cancer.

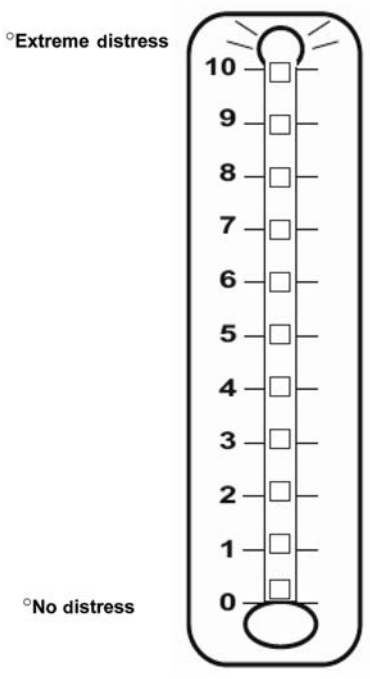
3. EVALUATION OF PSYCHOLOGICAL AND CANCER-RELATED SYMPTOMS

According to the IOM, screening, assessment, and treatment of psychosocial problems is now recommended as the standard of care in any oncology practice *(5)*. With the sixth vital sign being deemed "distress," the NCCN has developed the "Distress Thermometer" to screen for psychosocial problems in the oncology setting *(1)*. Although the distress thermometer has been described as having limitations *(61)* the instrument has been implemented in cancer centers across the country. For centers which have limited resources and would like to employ the distress thermometer in clinics treating HCC, we have modified this instrument to include additional psychosocial issues and symptoms specific for patients with HCC, see Table 1.

Furthermore, an additional, but not exhaustive, list of recommended instruments has been compiled to provide clinicians and researchers with a variety of methods to assess common presenting problems in HCC. Table 2 provides information regarding the number of items, scales, response scales, time frame, cutoff scores, and information regarding the reliability and validity of the instrument.

Table 1
Modified Distress Thermometer for Patients Diagnosed with Hepatocellular Carcinoma

Place a check mark next to the number (0–10) that best describes how much distress you have been experiencing in the past week including today:



Example: ☑

Please indicate if any of the following has been a problem for you in the past week including today. Be sure to check YES or NO for each, and place a check in the ☎ column if you want us to contact you to discuss this problem:

	YES	NO	☎
1. Practical problems			
Child care	☐	☐	☐
Housing	☐	☐	☐
Insurance/financial	☐	☐	☐
Transportation	☐	☐	☐
Work/school	☐	☐	☐
Communication to or between health care team	☐	☐	☐
Caregiving (e.g., child, parents, disabled family member)	☐	☐	☐
2. Family problems			
Dealing with children	☐	☐	☐
Dealing with partner (e.g., souse, girlfriend, boyfriend)	☐	☐	☐
Dealing with family of origin (e.g., mother, brother, grandmother)	☐	☐	☐
Are there cultural factors that may play a role in your medical treatment?	☐	☐	☐
3. Emotional problems			
Depression	☐	☐	☐
Fears (e.g.,needles, small spaces, blood, etc.,)	☐	☐	☐
Nervousness	☐	☐	☐
Sadness	☐	☐	☐
Worry	☐	☐	☐
Loss of interest in usual activities	☐	☐	☐
Do you have a personal history of depression?	☐	☐	☐
Stress/anxiety	☐	☐	☐
Use of alcohol	☐	☐	☐
Use of drugs (e.g., prescription or other)	☐	☐	☐
Use of tobacco	☐	☐	☐
Weight gain/loss	☐	☐	☐
4. Physical problems			
Appearance	☐	☐	☐
Bathing/dressing	☐	☐	☐
Breathing	☐	☐	☐
Changes in urination	☐	☐	☐
Constipation	☐	☐	☐
Diarrhea	☐	☐	☐
Eating	☐	☐	☐
Fatigue	☐	☐	☐
Feeling swollen or bloated	☐	☐	☐
Fevers	☐	☐	☐
Getting around/trouble getting out bed	☐	☐	☐
Indigestion	☐	☐	☐
Memory/concentration	☐	☐	☐
Mouth sores	☐	☐	☐
Nausea	☐	☐	☐
Nose dry/congested	☐	☐	☐
Pain	☐	☐	☐
Sexual	☐	☐	☐
Skin dry/itchy	☐	☐	☐
Sleep	☐	☐	☐
Tingling in hands/feet	☐	☐	☐
Change in taste	☐	☐	☐
Confusion	☐	☐	☐
Flushing	☐	☐	☐
Jaundice	☐	☐	☐
Not wanting to live	☐	☐	☐
Other:_____	☐	☐	☐

Table 2
Instruments to Assess Psychological Cancer-Related Symptoms in Adults with HCC

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
Depression							
Beck Depression Inventory (BDI-II) *(62–64)*	**21** Depression Score range: 0–63	4-point Likert Minimal depression:0–13 Mild depression:14–19 Moderate depression: 20–28 Severe depression: 29–63 *Past 2 weeks*	+**	+[b]	+	+	+
Center for Epidemiological Studies – Depression Scale (CES-D) *(65, 66)*	**20** Depressive symptomatology in cancer patients Score range: 0–60	4-point Likert Higher scores indicative of depression Mild depression: 16–26	+**	+[b]	+	+	+

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
		Major depression: ≥ 27					
		Past week					
The Hospital Anxiety and Depression Scale (HADS) *(67–70)*	**14** 1. Anxiety 2. Depression Score range (per scale): 0–21	4-point Likert **Subscale scores for anxiety or depression:** Normal: 0–7 Mild: 8–10 Moderate: 11–14 Severe: 15–21	+**	+[b]		+/–	
		Acute					
Patient Health Questionnaire – Depression Scale (PHQ-9) *(71–73)*	**9** Severity of depression	4-point Likert **Depression severity** None: 0–4 Mild: 5–9	+**	+[b]	+	+	+

(Continued)

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
	Score range: 0–27	Moderate: 10–14 Moderately severe: 15–19 Severe: 20–27 *Past 2 weeks*					
Anxiety							
State Trait Anxiety Inventory – Form Y (STAI) *(74–77)*	**40** 1. State anxiety 2. Trait anxiety Score range: 20–80	4-point Likert High scores on their respective scales mean more trait or state anxiety and low scores mean less *Acute and chronic*	+**/−	+[b]/−	+	+/−	+
The Hospital Anxiety and Depression Scale	**14** 3. Anxiety 4. Depression	4-point Likert **Subscale scores for anxiety or depression:**	+**	+[b]		+/−	

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
(HADS) *(67–70)*	Score range (per scale): 0–21	Normal: 0–7 Mild: 8–10 Moderate: 11–14 Severe: 15–21 *Acute*					
Quality of life							
Functional Adjustment to Cancer Therapy – Hepatobiliary Questionnaire[c] (FACT-Hep)*(78)*	**45** 1. Social/family well-being 2. Functional well-being 3. Physical well-being 4. Emotional well-being 5. Symptoms and side effects	5-point Likert High scores indicate higher quality of life *Past week*	+**	+[b]	+	+	

(Continued)

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
EORTC Quality of Life Questionnaire – Hepatocellular Carcinoma EORTC-QLQ -HCC *(79, 80)*	**48** 1. Functional • Physical • Role • Cognitive • Emotional • Social 2. Symptom • Fatigue • Pain • Nausea and vomiting 3. Global health 4. Quality of life Score range (per scale): 0–100	4- or 7-point Likert A high scale score represents a higher response level *Past week*		+[a]	+	+	+

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
Medical Outcome Study 36-item Short Form Health Survey (SF-36) *(81–83)*	**36** 1. Physical functioning (PF) 2. Role of limitations due to physical health problems (RP) 3. Bodily pain (BP) 4. General health perceptions (GH) 5. Vitality (VT) 6. Social functioning (SF) 7. Role of limitations due to emotional problems (RE)	Multiple response sets High scores indicates higher levels of functioning or well-being Standard: *4 weeks* Acute: *1 week*	+	+[b]	+	+	+

(Continued)

Table 2
(Continued)

Symptom	No. of items, scales, and score range	Response types, score cutoffs and time frame	Reliability		Validity		
			Temporal stability	Internal consistency	Content	Criterion	Construct
	8. General mental health (MH) Score range: 0–100						
EuroQoL (EQ-5D) *(84–87)*	**5 + VAS** 1. Descriptive index: • Mobility (MO) • Self-care (SC) • Usual activities (UA) • Pain /discomfort (PD) • Anxiety /depression (AD) 2. Visual analog scale (VAS)	Participants are asked to indicate their level of health by checking one of three boxes for each domain. For the visual analog scale, participants draw a line from a box to the point on the thermometer-like scale corresponding to their health state, 0–100 (100 = Best health state)	+	+[a]		+	+

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
Fatigue							
Revised Piper Fatigue Scale (R-PFS) *(88–91)*	**22** 1. Behavioral /severity 2. Affective meaning 3. Sensory 4. Cognitive/mood Score range: 0–10	11-point Likert **Severity of fatigue:** None: 0 Mild: 1–3 Moderate: 4–6 Severe: 7–10 *Acute*	+	+[b]	+	+	+
Functional Assessment of Chronic Illness Therapy (FACIT)-Fatigue scale Questionnaire[c]	**41** 1. Social/family well-being 2. Functional well-being	5-point Likert High score indicates higher quality of life, e.g., less fatigue	+	+[b]		+	

(Continued)

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
(FACT-F) *(91, 92)*	3. Physical well-being 4. Emotional well-being 5. Fatigue subscale	*Past week*					
Global Fatigue Index (GFI) *(91, 93)*	**15** Degree, severity, distress, and impact of fatigue Score range: 1–50	10-point Likert Scores range from 1 to 50 with higher scores indicating greater impairment from fatigue. *Past week*		+[b]		+	+
Multidimensional Fatigue Inventory (MFI-20) *(91, 94)*	**20** Phenomenology severity and impact of fatigue	7-point Likert Higher scores indicate a higher degree of fatigue	+	+[a]		+	+

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
	1. General fatigue 2. Physical fatigue 3. Mental fatigue 4. Reduced motivation 5. Reduced activity	*Previous days*					
Schwartz Cancer Fatigue Scale (SCFS) *(91, 95, 96)*	**28** Phenomenology and severity of fatigue 1. Physical 2. Emotional 3. Cognitive 4. Temporal Score range: 6–36	5-point Likert *Past 2–3 days*		+[b]	+	+	+

(Continued)

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
Fatigue Questionnaire (FQ) *(91, 97–99)*	**11** 1. Physical fatigue 2. Mental fatigue	4-point Likert and dichotomized scale Fatigue caseness is defined by a total dichotomized score of 4 or higher and a duration $\geq$ 6 months		+[b]		+	
Fatigue Symptom Inventory (FSI) *(91, 100, 101)*	**13** Intensity and duration of fatigue and its interference with quality of life Score range: 0–130	11-point Likert Higher scores indicate a higher degree of fatigue *Acute and past week*	–	+[b]			+

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
Sleep problems							
Pittsburgh Sleep Quality Index (PSQI) *(102)*	**11** 1. Subjective sleep quality 2. Sleep latency 3. Sleep duration 4. Habitual sleep efficiency 5. Sleep disturbances 6. Use of sleeping medication 7. Daytime dysfunction Score range: 0–21	4-point Likert Scores > 5 indicate poor sleep quality *Past month*	+*	+[b]	+	+	

(Continued)

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
Epworth Sleepiness Scale (ESS) *(103–106)*	**8** Measures daytime sleepiness in adults Score range: 0–24	4-point Likert Average: 7–8 Sleepy: > 10 Severe sleepiness: > 18 *Past 2 weeks*	+**	+[b]	+	+	
Pain							
Brief Pain Inventory (BPI) *(107–109)*	**23** 1. Pain intensity 2. Relief 3. Quality 4. Patients' perception of the cause of pain	11-point Likert Higher scores indicate deteriorated functional performance due to interference from pain *Acute, least pain, worst pain, and average pain over past week*	+**	+[b]	+		+

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
Multidimensional Pain Inventory (MPI) *(110, 111)*	**52** 1. Part I includes five scales designed to measure important dimensions of the chronic pain experience 2. Part II assesses patients' perceptions of the degree to which spouses or significant others' responses to their pain behaviors and complaints	7-point Likert	+*	+[a]/–	+	+/–	+/–

(Continued)

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
	3. Part III assesses patients' report of the frequency with which they engage in four categories of common everyday activities						
Visual Analog Scale (VAS) *(109, 112, 113)*	**1** Sensory intensity affective magnitude of pain Score range: 0–100	100-mm visual analogue scale Higher scores indicating higher levels of pain	+	+	+	+	+
Appetite/cachexia							
Functional Assessment of Anorexia/ Cachexia	**12** Anorexia/cachexia-related concerns	5-point Likert scale Higher scores indicate higher quality of life		+[a]		+	+

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
Therapy Subscale[d] (FAACT) *(114)*	Score range: 0–72	*Past week*					
Appetite and Diet Assessment Questionnaire (ADAQ)*(115, 116)*	**34** 1. Appetite 2. Change in appetite	Multiple response sets Decreased appetite ratings during the preceding week were evident primarily among patients who reported poor or very poor appetites				+	
Nausea and vomiting		*Acute and past week*					
Nausea Rating Index (NRI) *(117)*	**9** 1. Sensory 2. Affective	Ranking system Higher scores indicate worsening of nausea		+[b]		+	

(Continued)

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
	3. Evaluative 4. Miscellaneous						
Overall Nausea Intensity (ONI) *(117)*	**1** Score range = 0–5	Ranking system **Nausea intensity:** 0 = no nausea 1 = mild 2 = discomforting 3 = distressful 4 = horrible 5 = excruciating					
Cancer-related symptoms							
Memorial Symptom Assessment Scale (MSAS) *(118)*	**32** 1. Global distress index 2. Physical symptoms	4- or 5-point Likert scale Higher scores indicate higher levels of symptoms and distress		+[b]		+	

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
	Score range: 0–4.0 (averaged per scale)	3. Psychological symptoms *Past week*					
Multidimensional							
Four-Dimensional Symptom Questionnaire (4DSQ) *(119, 120)*	**50** 1. Distress (0–32) 2. Depression (0–12) 3. Anxiety (0–24) 4. Somatization (0–32) Score ranges: reported next to scales	Multiple response sets **Useful cutoff points:** Distress = 20 Depression = 4 Anxiety = 8 Somatization = 16 *Past week*		+[b]	+	+	+

(Continued)

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
Modified Distress Thermometer *(121)*	**61** 1. Distress thermometer 2. Practical problems 3. Family problems 4. Emotional problems 5. Physical problems	11-point Visual Likert scale and dichotomized (yes/no) scale Higher scores on visual distress thermometer indicate higher levels of distress *Acute and past week*				+	
Spiritual							
Spiritual Transformation Scale (STS) *(122)*	**40** 1. Spiritual growth 2. Spiritual decline	7-point Likert scale *Since diagnosis of cancer*	+**	+[b]		+/−	+

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
Functional Assessment of Chronic illness Therapy – Spiritual Well-Being Subscale[d] (FACIT-SP) *(123)*	**12** 1. Meaning/peace 2. Faith	5-point Likert scale High scores indicate higher quality of life *Past week*		+[b]	+	+	
Cognitive functioning							
Cognistat Neurobehavioral Cognitive Status Examination (Cognistat) *(124–127)*	1. Orientation 2. Attention 3. Comprehension 4. Repetition 5. Naming 6. Constructional praxis 7. Memory	Multiple response sets Two or more impaired scales indicate cognitive impairment *Acute*	–			–	+

(Continued)

Table 2
(Continued)

Symptom	*No. of items, scales, and score range*	*Response types, score cutoffs and time frame*	*Reliability*		*Validity*		
			Temporal stability	*Internal consistency*	*Content*	*Criterion*	*Construct*
	8. Calculations 9. Similarities 10. Judgment						
Mini Mental Status Exam (MMSE) *(128)*	**11** 1. Orientation 2. Registration 3. Attention 4. Calculation 5. Recall 6. Language Score range: 0–30	Multiple response sets Scores < 23 indicate cognitive impairment *Acute*	+			+	
Substance use							
Fagerström Test for Nicotine Dependence (FTND) *(129)*	**6** Score range: 0–10	5-point Likert scale and dichotomized (yes/no) responses		–	+	+	

Table 2
(Continued)

Symptom	No. of items, scales, and score range	Response types, score cutoffs and time frame	Reliability: Temporal stability	Reliability: Internal consistency	Validity: Content	Validity: Criterion	Validity: Construct
		Dependence scores: Very low: 0–2 Low: 3–4 Medium: 5 High: 6–7 Very High: 8–10					
		Acute					
Alcohol Use Disorders Identification Test (AUDIT) *(130, 131)*	**10** Score range: 0–40	3 or 5-point Likert scale Scores ≥ 8 indicate a strong likelihood of hazardous or harmful alcohol consumption.		+[a]		+	+
		Past year					

*$p \leq 0.05$; **$p \leq 0.001$
[a]$0.70 \leq \alpha \geq 0.80$; [b]$\alpha \geq 0.80$
[c]Combined with the FACT-G questionnaire
[d]Can be combined with the FACT-G questionnaire

Table 3
Assessment of Cancer-Related Symptoms

When did the symptom begin?
Has there been any change in the symptom over time?
How long does the symptom last once it begins?
How frequently does the symptom occur?
What factors improve the symptom?
What factors worsening the symptom (e.g., activity, inactivity)?
What meaning does this symptom or side effect have for you (e.g., pain means my disease is progressing)?
How severe is the symptom (does it impair social or functional status, mood)?
Have any medications improved or worsened the symptom?
Changes in appetite or weight associated with this symptom?
Do symptoms of anxiety or depression contribute to the exacerbation of this symptom?

Table 3 provides questions that may facilitate the assessment of common HCC-related symptoms and side effects such as pain, fatigue, or nausea and vomiting. Understanding the specific details of the onset, duration, and frequency of symptoms as well as factors that improve or worsen symptoms facilitates the clinician's ability to understand the potential symptom etiology and provide the most effective treatment. For mental health professionals working with HCC, Table 4 provides interview questions that may be useful in the psychosocial evaluation of patients diagnosed with HCC.

The most challenging aspect of diagnosis of a psychiatric disorder in cancer patients is the differential diagnosis of symptoms that may be a result of the cancer, other comorbid medical conditions, or medications. A careful medical history including a thorough understanding of the patients' current medication regimen is necessary. Differentiating psychiatric symptoms from symptoms associated with the cancer, liver disease, comorbid medical conditions, and medication side effects or interactions is imperative to make the appropriate diagnosis and treatment recommendations. Recommendations from a mental health professional may not result in a psychiatric diagnosis but further medical workup to rule out medical or medication-related symptoms.

3.1. Psychosocial Distress

A significant proportion of patients who are diagnosed with cancer have some level of psychological distress *(61)*. Patients diagnosed with liver

Table 4
Assessment of Psychosocial History

Problem	*Question*
	History
Sociocultural background	Where was the patient born and raised? Did the person emigrate from another country (if so, from where and at what age)? What ethnic or racial background patient identifies self? If the patient emigrated from another country, what is their level of acculturation? What is cultural meaning of their diagnosis or presenting problems? How would symptoms be treated in your culture?
Family	Family of origin Parents (past and current medical and psychiatric history, living, or deceased) Siblings (past and current medical and psychiatric history, living, or deceased) Current family (if applicable) Number of marriages Spouse/partner (medical, psychiatric history) Children (biological, step, foster, psychiatric and medical history, living with patient)
Education and occupation	Highest grade completed Difficulties or testing for developmental delays College or professional school Past and current occupations
Medical	Childhood or adolescent illness, surgeries, disabilities Adult illnesses, surgeries, disabilities Understanding of current illness and treatment
Current symptoms	Current symptoms Severity Frequency Duration Interference with social, occupational, or educational functioning Interference in specific situations Anything that improves or worsens symptoms Medications currently or prescribed for symptoms, adherence, response

(Continued)

Table 4
(Continued)

Problem	*Question*
Belief about symptoms and Illness	Beliefs regarding symptoms Understanding of illness, severity, prognosis, and treatment plan
Personal and family history of psychological disorders	Family history of psychiatric symptoms or disorders Personal history of psychiatric symptoms or disorders Pharmacological or psychological treatments Hospitalizations
	Current Context
Recent life events	Negative and positive events in life (home, work, school, relationships) Coping strategies use to manage stressors
Physical condition	Symptoms reflect current diagnosis
Drug and alcohol use	Past and current drug (recreational and prescription) Past and current tobacco use (cigarettes, pipes, chew) Past and current alcohol use Amount, frequency, duration, fluctuations in use, treatment, and response if indicated (for alcohol and drugs)
Intellectual and cognitive functioning	Intellectual strengths and deficits? Mental status (see below for mini-mental status)
Coping style	Adaptive or maladaptive coping strategies Coping successful in managing stress Short- versus long-term coping mechanisms
Sense of self and emotional expression	Feelings of self-worth Self-efficacy Expressed emotion
Religion and spirituality	Religious or spiritual affiliation or practice Is religious or spiritual practice important? Does spiritual or religious affiliation provide support?
	Resources and barriers
Individual resources	Factors the person views as integral to self Strengths s/he possesses How may strengths be used in treatment? How may weakness/strengths interfere with treatment?

Table 4
(Continued)

Problem	*Question*
Social resources (friends, family, and school/work)	Support Family Friends Work/school Quantity and quality of support Support increases or decreases stress
Community resources	Community resources available Community resources utilized Barriers to utilizing community resources Contributions to community
Behavior change	Stage of behavior change Barriers to behavior change (financial, educational, social) Beliefs about change in behavior (benefits, consequences)

cancer have been reported to have the third highest level of distress after lung and pancreatic cancer when compared to 14 other types of cancer *(3)*. Psychosocial distress as defined above would be expected when diagnosed with a potentially life-threatening illness such as cancer. Distress levels can vary depending on the diagnosis (e.g., lung or liver cancer) or expectations of treatment, which may be toxic, painful, and impair social, occupational/educational, cognitive, and/or physical functioning. Hepatocellular carcinoma carries with it a poor prognosis and although new treatments have become available and have promising results (e.g., Nexavar), the benefits of treatment remain modest *(132*, *133)*.

Not all patients express distress at the time of diagnosis. Some patients have excellent coping strategies and some patients may not present with observable levels of distress that impair functioning. Lack of distress may also be a result of the lack of understanding of the severity of the diagnosis. Furthermore, some patients and/or caregivers be in denial or use avoidant coping at the time of diagnosis. Denial or avoidance, if short lived, can be effective until a person can muster the resources to manage their emotions and begin to understand their options *(134)*. However, if a patient maintains denial and avoidant coping strategies for a long period of time, treatment may be delayed and the disease may progress as observed in other cancer types *(135–137)*.

Due to some risk factors associated with development of HCC (e.g., substance abuse/dependence), some patients may express feelings of guilt as in other cancer types in which behavioral factors may have contributed to the development of the disease *(138)*. In addition, family caregivers of patients with hepatobiliary carcinoma may have higher levels of anger and resentment when caring for these patients if they feel the "cause" of the cancer is a result of the patients' behavior. Although this has not been studied in HCC, research in other cancer types has discussed the role of anger in caregiving for patients diagnosed with cancer *(139*, *140)*. Patients may also express regrets for prior behaviors that lead to the development of their cancer. It is also not uncommon for childhood and adolescent issues (e.g., abuse, neglect) that may have contributed to the onset of risk behaviors *(141)* to surface at the time of diagnosis or over the course of treatment, as the patient may become increasingly vulnerable and dependent on professional and family caregivers.

The popular press has exaggerated the potential survival benefits of "fighting spirit" *(142–144)* and as a result, some patients diagnosed with cancer present to their healthcare professionals with a fear of expressing negative emotions (e.g., sadness, anger) as the patient believes it will result in progression of their cancer. It is critical for clinicians working with patients diagnosed with cancer to clarify the messages found in the popular media and to help them understand the benefits of expressing both positive and negative emotions and to normalize the experience of expressing positive and negative emotions in response to a diagnosis of cancer *(145*, *146)*.

Benefit finding or posttraumatic growth (PTG) has gained increased attention in oncology *(147)* and may be defined as "a positive cognitive process that is initiated to cope with traumatic events that extract an extreme cognitive and emotional toll." *(148)* In hepatobiliary carcinoma, 50% of patients report positive changes in their life after a diagnosis of cancer *(149)*. These results are consistent with previous research in other cancer types in which approximately half of the samples of patients reported positive as well as negative changes after a diagnosis of cancer *(148*, *150)*. However, patients with hepatobiliary carcinoma reported a lower mean PTG score than breast cancer patients *(149)* which may be secondary to differences in prognosis or gender differences observed in HCC versus breast cancer. A 2:1 gender ratio (male to female) exists in HCC whereas the majority of patients with breast cancer are female. Prior research has demonstrated that females tend to have higher PTG scores than males *(148*, *149*, *151)*. Patients diagnosed with HCC who reported higher levels of PTG were also found to have better immune system functioning *(152)*. Further research is warranted in understanding the construct (definition), process, and health outcomes associated with PTG.

4. COMMON PRESENTING PROBLEMS IN HCC

4.1. Psychological Disorders and Treatment Recommendations

In addition to the emotional and psychological reactions to the diagnosis of cancer as described in the previous section, persistent psychological distress may exacerbate previous psychiatric disorders in remission and a diagnosable disorder may develop. Secondary to the primary risk factors associated with HCC (substance abuse/dependence), patients with a diagnosis of HCC may have a greater likelihood of presenting with psychiatric distress or comorbid psychiatric disorders such as mood or anxiety disorders. In a minority of patients, psychiatric symptoms/disorders may develop for the first time with the stress of the diagnosis, treatment, and poor prognosis often associated with HCC. Below is a brief introduction to some of the most common presenting psychiatric disorders in patients with HCC. The chapter will provide a brief overview of the diagnosis and treatment of these disorders for both the medical and mental health professional. Since a paucity of research exists in regard to psychosocial issues in HCC, the majority of the research referenced will be in regard to research that has been conducted in cancer patients more broadly.

Psychological Disorders Common in Patients with Hepatocellular Carcinoma
Adjustment disorder
Depression
Phobias and anxiety
Substance dependence

4.2. Adjustment Disorder

Adjustment disorder is the most frequently diagnosed psychological disorder in cancer patients *(1)*. Adjustment disorder, according to the *Diagnostic and Statistical Manual of Mental Disorders IV* may be defined as the "development of emotional or behavioral symptoms in response to an identifiable stressor(s) occurring within three months of the onset of the stressor(s)." *(153)* The symptoms must develop within 3 months of the onset of the stressors and cause marked distress which is considered in excess of what would be expected, and also result in significant social, occupational, or education functioning *(153)*. In a study of a mixed sample of cancer patients, 15% of patients (49% of all psychiatric diagnoses) met the DSM-IV criteria for adjustment disorder with depressed or anxious

mood. Although there have been mixed results regarding the predictors of adjustment disorder, a combination of factors including disease (e.g., stage of cancer) and treatment-related factors (e.g., chemotherapy), awareness of diagnosis and prognosis, and social support have been reported *(134)*. At diagnosis, patients with HCC often have advanced disease (stage III and IV) and poor prognosis and therefore may have greater distress than other cancer types *(3)*. Treatment recommendations for adjustment disorder with symptoms of depressed mood and/or anxiety are similar to recommendations outlined below for the treatment of major depressive disorder and generalized anxiety disorder.

4.3. Major Depressive Disorder

Depression has received the greatest attention in regard to research in patients diagnosed with cancer. It is difficult to reach definitive conclusions regarding the prevalence of depression in cancer patients due to the variation in the definition and measurement of depression, and timing of assessment across studies. A recent review suggested that 0–38% of patients diagnosed with cancer may meet the DSM-IV criteria for major depressive disorder (MDD) *(154)*. An additional 20% may meet the criteria for depression spectrum disorders *(154)*. For a complete review, Massie provides a summary of the prevalence rate according to cancer type, method of measurement, and timing of assessment *(154)*.

Major depressive disorder (MDD) should be differentiated from an adjustment disorder with depression. In MDD, the number of symptoms required (five or more) and the duration of these symptoms (2 weeks or longer for MDD) differ from adjustment disorder. Symptoms of MDD may include (1) persistent sad, anxious, or "empty" mood; (2) loss of appetite and/or weight loss or conversely overeating and weight gain; (3) insomnia, early morning awakening, or oversleeping; (4) restlessness or irritability; (5) psychomotor agitation or psychomotor retardation; (6) feelings of worthlessness, inappropriate guilt, or helplessness; (7) feelings of hopelessness or pessimism; (8) difficulty thinking, concentrating, remembering, or making decisions; (9) thoughts of death or suicide or attempts at suicide; (10) loss of interest or pleasure in hobbies and activities that were once enjoyed; (11) withdrawal from social situations, family, and friends; and/or (12) decreased energy or fatigue *(153)*.

The treatment of depression in cancer is critical as several studies have now reported a link between depressive symptoms and increased cancer-related mortality *(7, 155–163)*. A recent study of patients diagnosed with HCC observed that 37% of patients reported depressive symptoms in the clinical range of the Center for Epidemiological Studies – Depression (CES-D) scale at the time of diagnosis *(7)*. Moreover, elevated depression

scores on this measure predicted reduced survival. Patients who had vascular invasion with high depression levels survived 5.2 months compared to an average of 11 months survival in patients with lower depression scores *(7)*. Among patients without vascular invasion, those with elevated scores survived 17 months versus 27 months for those with lower depression scores *(7)*, see Table 5.

Table 5
Cox Regression Analysis of Sociodemographic, Disease-Specific Variables, and Depressive Symptoms Affecting Survival (*N*=101)

				95% CI	
Variable	B *(SE)*	*Wald*	p *level*	*Lower*	*Upper*
Diagnosis					
HCC		0.488	0.92		
CCC	0.001 (0.465)	0.001	0.99	0.402	2.491
NET	−0.021 (0.876)	0.001	0.98	0.176	4.454
METS	0.575 (0.943)	0.371	0.54	0.280	11.286
Gender					
Male/female	0.417 (0.359)	1.355	0.24	0.752	3.066
Age		0.009	0.99		
<50	−0.046 (1.131)	0.002	0.97	0.104	8.761
≥50	−0.072 (1.115)	0.004	0.95	0.105	8.278
Ethnicity					
Caucasian/non	−0.219 (0.434)	0.256	0.61	0.289	3.567
Hepatitis					
B and/or C/none	−0.129 (0.330)	0.152	0.70	0.460	1.680
Cirrhosis					
Present/absent	−0.569 (0.330)	2.333	0.13	0.272	1.275
Tumor size					
<5 cm/>5 cm	0.295 (0.332)	0.789	0.38	0.700	2.576
Lesion number					
<3/≥3 lesions	−0.205 (0.266)	0.595	0.44	0.483	1.373
Vascularity					
Hyper or mixed/hypo	−0.216 (0.399)	0.292	0.59	0.368	1.763
Vascular invasion					
Present/absent	1.409 (0.337)	17.517	0.001	2.116	7.918
CES-D					
<16/>16	0.648 (0.297)	4.771	0.029	1.069	3.422

HCC=Hepatocellular carcinoma; CCC=Cholangiocarcinoma; NET=Neuroendocrine carcinoma of the liver; METS=Colorectal carcinoma with liver metastases; HIA=Hepatic

Furthermore, two studies have now suggested that biological changes associated with the cancer may contribute to the development of depressive symptoms even before the cancer is diagnosed *(164, 165)*. Research is underway regarding the role of underlying biological mechanisms that may be associated with depression and other cancer-related symptoms (e.g., pain, fatigue) in HCC. It is likely that there are at least two different types of depression in people diagnosed with HCC. Depression may be a part of a cluster of symptoms characterized by "sickness behavior" which includes feelings of malaise, social withdrawal, fatigue, pain, difficulty sleeping, and decreased intake of food and liquids. This type of depression may be associated with biological changes (e.g., hormones, cytokines) that may be a result of the tumor growth. Upon evaluation, a predominance of somatic symptoms may be observed (e.g., changes in appetite, sleep, fatigue). Whereas, a second type of depression may result from an accumulation of stressors and lack of resources (e.g., social support, effective coping strategies). Depression in this type of patient may be characterized by the report of greater emotional (e.g., sadness) and/or cognitive symptoms (e.g., difficulties concentrating, suicidal ideation) associated with depression.

As with depression in a psychiatric setting, the most effective treatment for depression in medically ill populations, including cancer, includes a combination of pharmacological treatment and psychotherapy (e.g., cognitive-behavioral or interpersonal) *(166, 167)*. To reduce the risk of relapse of depressive symptoms, a minimum of 6 months of treatment is recommended. To prevent recurrence in patients who report two or more episodes in 5 years, long-term antidepressant medication may be recommended *(168, 169)*. Psychosocial interventions are also being developed to reduce distress and depression in patient with cancer *(170–173)*. A randomized controlled trial is currently underway to test the efficacy of a collaborative care intervention in the treatment of cancer-related symptoms in patients with hepatobiliary carcinoma *(173)*.

4.4. Anxiety Disorders and Phobias

Like depression, anxiety is also an important factor in cancer treatment, as these symptoms can affect adherence to medical treatments *(174)*. Studies have previously found that approximately 44% of patients reported some level of anxiety and 23% of patients reported significant anxiety that impaired functioning *(175, 176)*. The most common anxiety disorder observed in patients with cancer may be generalized anxiety disorder (GAD). Generalized anxiety disorder is defined as excessive worry occurring more days than not for a period of at least 6 months *(153)*. The person with GAD has difficulty controlling the worry, and it is often associated with three or more of the following symptoms: (1) restlessness or

feeling keyed up or on edge; (2) being easily fatigued; (3) difficulty concentrating or mind going blank; (4) irritability; (5) muscle tension; and (6) sleep disturbances. The anxiety must cause marked impairment in social, occupational, or educational functioning *(153)*.

Anxiety may be related to both cancer and non-cancer-related cognitions that may be affected by the disease and treatment. Cognitions that can contribute to anxiety may include fear of recurrence, apprehension regarding receipt of results concerning their response to treatment (e.g., CT scan results), anxiety regarding painful or uncomfortable medical procedures, and fear of death and symptoms at the end of life. In addition, patients may also have other non-cancer-related worries that should be identified and treated (e.g., finances, caregiving, child care, transportation difficulties).

Posttraumatic stress disorder (PTSD) is defined as an extreme traumatic event that includes actual or perceived threat to life or serious injury. An individual must experience intense fear, helplessness, or horror as a result of the event and meet the criteria for three categories of symptoms following the event including re-experiencing, avoidance, and physiological arousal. The symptoms must persist for at least 1 month and result in a marked impairment in social, occupational, or educational functioning. The prevalence of PTSD in cancer patients has been found to range from 16 to 32% *(177*, *178)*. Limitations of previous research include the lack of assessing traumatic events and PTSD prior to the diagnosis of cancer. Veterans may be overrepresented in samples of patients diagnosed with HCC secondary to the risk factors associated with HCC (e.g., substance dependence) and therefore are more likely to present with a current or past history of PTSD when compared to other cancer types *(179–181)*.

In other cancer types, predictors of PTSD include dissociative symptoms, greater distress at the time of diagnosis, prior negative life stressors *(182)*, a history of psychological problems, female gender *(183)*, younger age at diagnosis *(184*, *185)*, lower socio-economic status *(184)*, lower education *(184*, *186)*, avoidant coping style *(187)*, low social support *(187*, *188)*, and reduced physical functioning *(186)*. Although the treatment of PTSD in other populations has been extensively reported *(189–193)*, no study to our knowledge has tested the efficacy of interventions to treat PTSD in patients diagnosed with cancer. Although not tested, behavioral and pharmacological treatments that have been demonstrated to be effective in the general population may also be effective in patients diagnosed with HCC, however this warrants further research.

Panic disorder may be characterized by a series of intense periods of extreme anxiety and somatic symptoms including shortness of breath, tachycardia, dizziness, chest pain, trembling, chills, and fear of dying or going crazy *(153)*. The attacks may last a few minutes to hours and often come on suddenly. To meet the DSM-IV criteria the person must have had at least one

attack in the past month and continue to have (1) persistent concern about additional attacks, (2) worry about the implications of the attacks or the consequences of the attacks, or (3) significant behavior change related to the attacks *(153)*.

If panic attacks develop in the context of a diagnosis of HCC, it is often secondary to an exacerbation of a previous history of panic disorder that may or may not have been treated. Slaughter and colleagues reported that prevalence of panic attacks was approximately 20% in a sample of hospitalized cancer patients *(194)*. The stress associated with the life-threatening disease and the nature of treatment may exacerbate panic disorder that may have been in remission or exacerbate the frequency of attacks. People with panic disorder also report agoraphobia (fear of places or situations from which escape may be difficult or embarrassing or in which help may not be available). Agoraphobia itself may have a significant effect on the ability of a patient to remain in medical treatment if the panic attacks and agoraphobia are untreated.

Finally, common fears and phobias that did not previously interfere with functioning may become problematic if not identified and treated. Fears and phobias, particularly of needles, or claustrophobia may result in delayed or early termination of treatment. A diagnosis of a specific fear includes marked and persistent fear that is excessive or unreasonable and may be cued by the presence or anticipation of the specific object or situation *(153)*. The phobic stimulus is often avoided or endured with great anxiety. The avoidance or anxious anticipation often interferes with the individual's social, occupational, or educational functioning *(153)*. Research has been conducted in regard to stress-reducing medical devices as well as cognitive-behavioral therapies to reduce anxiety or treat phobic reactions *(195)*.

4.5. Substance Abuse/Dependence

The distinction between substance abuse and dependence is rarely defined outside of psychiatry. Substance *abuse* may be defined as the use of a substance on repetitive occasions that results in the failure to fulfill major obligations in social, occupational, or educational settings *(153)*. The substance use may also result in an individual being involved in physically hazardous situations and/or be associated with legal problems. The abuse of a substance or substances continues despite recurrent social or interpersonal problems. In contrast, substance *dependence* refers to the pattern of substance use that results in impairment or distress of three or more of the following areas in a 12-month period: (1) development of tolerance—marked increase in the amount of substance needed to achieve the desired effect or diminished effect with use of same amount *(153)*; (2) withdrawal or continued use of substance to avoid withdrawal symptoms; (3) the substance taken in larger

amounts or over longer periods than intended; (4) persistent desire or unsuccessful efforts to decrease use; (5) a great amount of time spent obtaining substances or recovering from its effects; (6) important occupational, social, or recreational activities are reduced due to substance use; and (7) substance use is continued despite psychological or physical problems *(153)*. Whether the individual currently uses drugs or if they have a distant history of drug abuse or dependence, continued evaluation and treatment of substance use when indicated is imperative secondary to the high relapse rates observed in substance abuse and dependence *(196*, *197)*.

Similar to drug abuse and dependence, persons who have a history of alcohol abuse or dependence have the risk of relapse, particularly when facing major life stressors *(198*, *199)*. All patients with chronic liver disease, not only those with alcohol-related HCC, should be evaluated for current alcohol use, as alcohol and drugs have also been found to have a synergistic effect with HCV in the development of cirrhosis and may increase the rate of disease progression *(200*, *201)*.

Identifying whether a patient is diagnosed with substance abuse and/or dependence is critical to the immediate care of the patient. If the patient has an active alcohol abuse or dependence disorder, assistance with addiction counseling may be essential to their initial stabilization and their ability to participate in treatment planning and adherence to the cancer therapy regimen. The clinician's efficacy in assisting the patient will largely depend on the stage of contemplation and insight of the patient, whether they acknowledge their addiction problem and are willing to seek treatment and have recruited a stable support system of family and friends. Consultation by a mental health professional can establish the correct psychiatric diagnosis and provide recommendations for appropriate treatment options. In addition to the immediate benefits of abstinence, if the patient plans to undergo surgical treatment options for the cancer (e.g., resection, radiofrequency ablation, or transplantation), active alcohol use has been demonstrated to result in surgical complications including cognitive impairment *(201)*, increased rates of pulmonary complications *(28*, *202–204)*, and infection *(202*, *203*, *205)*. Effective interventions for excessive alcohol consumption have been reported and tailored interventions have been effective prior to elective surgery *(206)*.

Approximately 55% of the general population has a lifetime history of tobacco use *(153)*. Tobacco use has been found to be high in patients diagnosed with HCC *(207)*. For patients diagnosed with lung cancer that continued smoking after diagnosis, increased mortality and reduced response to chemotherapy were reported *(50–52)*. If surgical intervention is indicated, smoking cessation prior to surgery is recommended as tobacco use has been found to be associated with a number of surgical complications including increased risk for infection *(208)*, slowed wound healing *(209*, *210)*,

pneumonia *(204)*, poor outcomes after transplantation *(29*, *210)*, pulmonary complications *(28*, *212)*, and vascular complications *(30)*. Smoking cessation at least 6–8 weeks prior to surgery has been suggested to improve immune functioning and wound healing and reduce overall perioperative morbidity *(213–217)*.

5. CANCER-RELATED SYMPTOMS AND TREATMENT RECOMMENDATIONS

The National Institute Consensus statement on "Symptom Management in Cancer: Pain, Depression and Fatigue" concluded that the three most common and untreated cancer-related symptoms were pain, fatigue, and depression *(218)*. Approximately 40% of patients at the time of diagnosis reported pain *(207)*, 15% reported weakness or malaise *(207)*, and 37% depressive symptoms *(7)*. These cancer-related symptoms, as well as others, if left untreated can significantly impair patient's quality of life and may delay or prevent treatment. Increasingly, the co-variation of cancer-related symptoms is being studied in regard to the common underlying biological mechanisms *(219)*. Novel treatments are currently being tested to treat comorbid symptom rather than each symptom independently *(219)*. Although patients diagnosed with HCC may experience numerous symptoms and side effects from treatment, we will review the most common symptoms and side effects that impair psychosocial functioning in patients with HCC and a brief overview of pharmacological and behavioral treatments recommendations.

Physical Symptoms Common with Hepatocellular Carcinoma
Fatigue
Pain
Sleep problems
Nausea and vomiting
Sexual dysfunction
Cognitive impairment

5.1. Fatigue

Fatigue is one of the most common and debilitating symptoms for people diagnosed with cancer. Fatigue can be associated with the disease as well as treatments for HCC. According to the NCI, fatigue occurs in 14–96% of people with cancer *(220–223)*. Fatigue is one of the most difficult disease-related side effects to treat and can be acute or chronic. The

etiologies include biological (e.g., anemia, tumor necrosis factor, chemotherapy or radiation, changes in metabolism or hormones), psychological (e.g., depression, stress), and/or behavioral factors (e.g., sleep disturbance, pain) and are often difficult to disentangle *(220, 224–230)*. Patients often report that fatigue results in higher levels of distress than pain due to the impairment of occupational, educational, and interpersonal functioning as well as financial losses. Treatment of fatigue depends on the underlying etiology. Based on the contributing factor(s), different interventions may include changes in medication regimens, red blood cell transfusions, increasing physical activity, treating depressive symptoms, treatment of anemia, or administration of psychostimulants *(231–248)*. Behavioral interventions such as improved nutrition as well as energy conservation and restoration activities may also be recommended *(249–252)*.

5.2. Pain

Approximately 40% of patients diagnosed with HCC report upper quadrant abdominal pain and/or pain in their shoulder at the time of diagnosis *(208)*. Chemoembolization or surgery can result in transient pain and may be treated effectively with opioids. In advanced stages of the disease, ascites can cause pain and discomfort. Although not all patients report pain associated with the disease or treatment, for the patients who do report these symptoms, evaluation and appropriate treatment is warranted as the pain often significantly interferes with functioning. Pain is assessed through self-report and/or interview methods (Table 3). Several standardized instruments that are useful in measuring pain in a clinical or research setting, including the most commonly employed measure of pain, the visual analog scale.

Pharmacological interventions are often the first line of treatment in cancer-related pain due to the severity, particularly in HCC. However, behavioral strategies can also complement the management of pain with medications. Managing pain in the patient with chronic liver disease is challenging for several reasons. A large percentage of patients may have a history of substance abuse or dependence making both patients and health providers reluctant to prescribe narcotics which are often the treatment of choice for cancer-related pain *(253–257)*. With increased regulation of narcotics, healthcare providers have become increasingly reluctant to prescribe narcotics and as a result patients' pain is often undertreated *(258–264)*. Furthermore, use of narcotics as well as other medications in the context of cirrhosis should be prescribed cautiously, as research has demonstrated that there are differences in metabolism of these drugs in the cirrhotic liver *(265, 266)*. It is recommended that medications be taken on a regular schedule to maintain a therapeutic dose, as pain becomes more difficult to reduce if the pain reaches high levels. It should also be noted that long-term treatment of

pain with narcotics will result in increased tolerance and that the healthcare provider should be aware that patients will request higher doses over time, and should not necessarily be recognized as drug seeking. Unfortunately, as doses are increased the side effect profile also may worsen (e.g., increases in nausea, constipation, or changes in mental status) and patients may have increasing difficulties managing the side effects which may also result in other symptoms (e.g., constipation and pain).

For long-term treatment of pain, it has been recommended to change medications occasionally to decrease tolerance *(267–270)*. Multiple delivery systems (e.g., orally, as suppositories, and intravenously) have been developed to make certain pain be effectively managed independent of other symptoms or side effects (e.g., nausea/vomiting) or at the end of life when oral medication may not be tolerated. The role of the mental health professional often includes the evaluation of the pain and feedback to the healthcare professionals involved in the pharmacological management of the pain as well as how a patient's prior history may affect patient or healthcare provider perceptions associated with pain management. Issues of adherence are of particular importance when addressing management of pain. Understanding the meaning the patient attributes to the pain is also important, as some individuals may view the pain as a response to treatment while others view the pain as a sign of disease progression.

In addition to pharmacological interventions for pain, several behavioral strategies may also be employed to alleviate pain. First, the treatment of depression and/or anxiety has been demonstrated to reduce the perception or sensation of pain *(271–275)*. Relaxation techniques such as progressive muscle relaxation or autogenics are most often employed to treat pain *(276–280)*. Heat or cold packs are also used to decrease pain as well as massage, pressure, and vibration *(281)*. In some instances, exercise and/or frequent changes of position may be recommended based on the type of pain the patient presents *(281)*. If the pain persists, invasive treatments including nerve blocks and surgical interventions are available to patients.

5.3. Sleep Problems

While sleep disorders occur in 12–25% of the general population *(282)* it is estimated that 45% of cancer patients experience sleep disturbance *(230*, *283*, *284)*. In an unpublished study, 80% of patients with hepatobiliary carcinoma reported a disruption in sleep at the time of diagnosis. The most prevalent sleep disorders in the general population, and also in people diagnosed with HCC include insomnia, sleep apnea, and restless legs syndrome *(285*, *286)*. Several factors may contribute to insomnia including anxiety at diagnosis, fear of recurrence, pain, hospitalization, fatigue, and disturbance of sleep–wake cycle as a result of treatment and/or side effects, and changes in gastrointestinal and genitourinary functioning *(284*, *287)*. Medications

including vitamins, corticosteroids, neuroleptics, stimulants, sedatives and hypnotics, anticonvulsants, and sympathomimetics may result in sleep disturbances *(287)*. Poor sleep quality results in poor day time functioning and performance and increased risk for developing delirium, anxiety, and depression and reduced ability to manage stress *(287–289)*.

Evaluation and treatment of sleep disorders may include a screening by a healthcare professional and, if necessary, follow-up with a specialist at a sleep disorders center if a sleep disorder is suspected. If the sleep disturbance is amenable to behavioral intervention (e.g., change in wake–sleep schedule, improve sleep hygiene) or changes in pharmacological regimen (e.g., decrease dose of pain medication, eliminate medication causing insomnia), the need for further evaluation may not be necessary. However, if the insomnia persists or if it is suspected that the patient may have sleep apnea, restless legs syndrome, or other sleep disorders (e.g., REM sleep disorder) a referral to a sleep disorders center may be recommended.

Although benzodiazepines are often prescribed to treat insomnia, in patients with cirrhosis the difference in the metabolization of these drugs suggests that the patient should be started on a reduced dose *(265*, *266)*. For any patient, the use of benzodiazepines for more than 2 weeks is not recommended due to psychological or physical dependence *(283*, *284)*. However, the advantages and disadvantages of using sleep aids should be weighed as sleep deprivation may also have negative health and psychological consequences *(288*, *290*, *291)*. Behavioral interventions for insomnia include stimulus control and sleep hygiene techniques *(284*, *290–298)*, relaxation techniques *(292)*, as well as cognitive-behavioral strategies to reduce anxiety or fears may also be effective in decreasing insomnia *(292)*. Table 6 provides recommendations from the National Cancer Institute regarding sleep hygiene strategies specifically for patients diagnosed with cancer *(293)*.

Sleep apnea can be classified into central and obstructive sleep apnea. Central sleep apnea may be diagnosed when the central nervous system fails to send the appropriate signals to the breathing muscles to initiate respirations. Obstructive sleep apnea is a result of the lack of air flow into or out of the person's nose or mouth *(299*, *300)*. Obstructive sleep apnea is more common and the person is often reported by others to snore or gasp for breath during sleep. These periods of lack of breath can occur hundreds of times per night and cause excessive daytime sleepiness. Although a higher rate of sleep apnea has been observed in head and neck cancer patients as a result of anterior mandibulectomy, the prevalence of sleep apnea may be higher than the general population in hepatocellular carcinoma, particularly in NASH-related HCC, in which obesity is often comorbid with HCC. As a result, careful evaluation of sleep and wake disturbances and appropriate referral to a sleep disorders center is recommended.

Table 6
Sleep Hygiene for the Cancer Patients

Sleep hygiene practice specific for cancer patients	*Keeping the patient's skin clean and dry*
	Giving back rubs and/or massaging areas of the body to bring comfort to the patient (e.g., bony prominences, head and scalp, shoulders, hands, and feet)
	Keeping bedding and/or surfaces of support devices (chairs and pillows) clean, dry, and wrinkle-free
	Ensuring adequate bedcovers for warmth
	Regulating fluid intake to avoid frequent awakening for elimination
	Encouraging bowel and bladder elimination before sleep
	Promoting optimal bowel function (increased fluids, dietary fiber, and use of stool softeners and laxatives)
	Using a condom catheter for nocturnal incontinence
	Providing a high-protein snack 2 hours before bedtime (e.g., milk, turkey, or other foods high in tryptophan)
	Avoiding beverages with caffeine and other stimulants, including dietary supplements that promote metabolism changes and appetite suppression
	Encouraging the patient to dress in loose, soft clothing
	Facilitating comfort through repositioning and support with pillows as needed
	Encouraging exercise or activity not less than 2 hours before bedtime
	Encouraging the patient to keep regular bedtime and awakening hours
	Minimizing and coordinating necessary bedside contacts for inpatients

Restless legs syndrome is an uncomfortable sensation in the legs that is often described as a crawling, tingling, pulling, or twitching sensation that occurs when a person is sitting or lying down. The individual often has the urge to move to relieve the sensation. The symptoms usually worsen

in the evening and may be painful. It is estimated that one in ten persons are affected by this syndrome. Although to the authors' knowledge no study has been conducted in patients with HCC, restless legs syndrome is observed clinically, but it is not clear if there are higher rates in patients diagnosed with HCC than in the general population. If restless legs syndrome is suspected, a referral to a sleep disorders clinic may be recommended *(301)*.

5.4. Nausea and Vomiting

Nausea may be defined as an unpleasant wave-like feeling at the back of the throat or in the stomach that may involve the forceful elimination of the contents of the stomach *(302)*. For patients diagnosed with hepatocellular carcinoma, a loss of appetite and nausea may be associated with the disease. In addition, several of the chemotherapy agents utilized to treat hepatocellular carcinoma (e.g., Cisplatin, Gemzar, Oxaliplatin) have varying levels of emetic effects *(303–305)*. Immediate treatment of nausea and vomiting is imperative as it can greatly interfere with the patient's ability to receive treatment and can result in other medical complications (e.g., dehydration, Mallory Weiss tear, broken bones, electrolyte imbalance) *(306*, *307)*. Nausea and vomiting can be classified into four different categories including acute, delayed, anticipatory, and chronic.

Mental health professionals can facilitate the assessment of nausea and vomiting through their contact with patients between visits with healthcare providers but also intervene behaviorally to facilitate the response to the anti-emetics that are given prophylatically as well as subsequent to treatment. The mental health professional can also assess the type of nausea the patient may be experiencing as well as the potential predictors (e.g., constipation, anxiety). In the case of anticipatory nausea and vomiting, the mental health professional may play a greater role, as the nausea is a conditioned response that may be treated with behavioral intervention *(308–312)*. Anticipatory nausea is the conditioned response of an odor, food, setting, or event in which the person experienced chemotherapy-related nausea. The pairing of chemotherapy-induced nausea and the stimulus results in the conditioned response of nausea and vomiting to the new stimulus (e.g., food, setting). When the patient is presented with the stimulus in the absence of the chemotherapeutic agent, s/he will develop nausea and even vomiting.

Predictors of anticipatory nausea may include (1) being younger than 50 years of age; (2) female; (3) severity of nausea and vomiting after the last chemotherapy session; (4) feeling warm or hot after the last chemotherapy session; (5) a history of motion sickness; (6) feeling dizzy or lightheaded after chemotherapy; (7) sweating after the last chemotherapy session; (8) experiencing weakness after the last chemotherapy session; (9) having a high level of anxiety; and (10) having morning sickness during pregnancy.

Systematic desensitization is one of the most effective treatments for anticipatory nausea and vomiting *(313–315)*. In regard to the other types of nausea, behavioral treatments may facilitate the effectiveness of the anti-emetic medications but the results of behavioral intervention alone have received mixed results in regard to their effectiveness with immediate, delayed, or persistent nausea and vomiting *(316, 317)*.

5.5. Sexual Dysfunction

Sexual problems have been studied in patients with cancer of the reproductive organs and found to be higher than the general population *(318–321)*. Although little research has been conducted regarding sexual dysfunction in HCC, a recent study reported the prevalence of sexual dysfunction to be approximately 25% *(322)*. Andersen, in an excellent review, found that individual self-schema (image of self), psychiatric and medical symptoms, psychological/behavioral status, and extent of disease and treatment, contributed to increased rates of sexual dysfunction in people diagnosed with cancer *(323)*. People diagnosed with HCC would be expected to report higher levels of sexual dysfunction secondary to (1) neuroendocrine changes that result from the disease and/or treatment; (2) changes in body image associated with gynecomastia, cachexia, and ascites; (3) high level of comorbid medical conditions that may result in increased sexual morbidity (e.g., diabetes); (4) medications that result in sexual side effects (e.g., narcotics, antidepressants, benzodiazepines, hypertension medications); (5) cirrhosis; and (6) comorbid psychological symptoms (e.g., depression, anxiety) *(322)*.

Zifroni and colleagues reported that men with chronic liver disease (CLD) and a Child-Pugh score of B or C reported higher rates of sexual dysfunction and significant reductions in free testosterone levels *(324)*. In a recent study with patients diagnosed with HCC, no difference was found in those men who had Child Pugh B or C scores and rates of sexual dysfunction when compared to those who had a Child A score in both patients with HCC and CLD *(322)*. No demographic or other disease-specific variables including age, ethnicity, etiology of disease, or cirrhosis were found to be associated with increased rates of sexual dysfunction in HCC *(322)*. The high rates of sexual dysfunction in HCC patients were found to be secondary to medical conditions and medications associated with increased sexual morbidity *(322)*. Patients with HCC and CLD had a number of medical conditions including hypertension, diabetes, cardiovascular disease, and depression, in which the disease and treatments are commonly known to cause sexual problems.

Serum testosterone levels have been found to be reduced in patients receiving chronic opioid therapy *(325)*. Neuroendocrine changes that may

be associated with the disease and/or treatment of hepatobiliary disease may also contribute to the increased sexual morbidity in these populations including changes (1) in metabolic clearance rates; (2) in plasma production and total and free levels of testosterone; (3) reduced testosterone responses to human chronic gonadotropin stimulation; (4) in estradiol and lutenizing and follicle-stimulating hormone levels; and (5) in binding capacities of sex steroid-binding globulin *(326–330)*.

Psychiatric symptoms such as anxiety, depression, and substance abuse or dependence may be associated with sexual dysfunction *(3)*. Van Lankveld and Grotjohann concluded that people reporting a sexual problem have higher rates of lifetime depression and anxiety *(331)*. Furthermore, chronic alcohol use has been associated with male erectile dysfunction *(332)*. In the recent study of patients with HCC, individuals who reported a sexual problem and/or met the criteria for a DSM-IV diagnosis for a sexual disorder had a lower emotional well-being *(333)*. It is not known whether the increased psychological distress was a result of the sexual dysfunction or whether the psychological distress contributed to the sexual problems. The study concerning sexual dysfunction in patients with HCC, however, does suggest that patients with sexual dysfunction have lower health-related quality of life (HRQL) than patients without sexual dysfunction and warrants treatment.

Patients have differing levels of interest in regard to the evaluation and treatment of sexual dysfunction in the context of cancer. For some patients (and partners), continued sexual activity is important for their relationship while for others sexual activity may not be considered important, and despite impairment in sexual functioning are not interested in pursuing evaluation or treatment. If evaluation is recommended, both the patient and sexual partner will be involved in the diagnosis and treatment. Treatment of sexual dysfunction involves the differential diagnosis of the etiology which may include disease-, treatment-related, or psychiatric factors. It is recommended to rule out medical causes of sexual dysfunction prior to treatment of psychiatric factors that may be contributing to the impairment. Although the scope of the book does not permit a full description of each of the potential treatment options for the numerous male and female sexual dysfunctions (e.g., dysperenia, erectile dysfunction), excellent resources are available from the National Cancer Institute and the American Cancer Society (ACS) *(334–336)*. A comprehensive evaluation by a specialist (e.g., urologist, gynecologist) may be recommended to facilitate the appropriate diagnosis and treatment.

5.6. Cognitive Impairment

Delirium may be defined as "a disorder of global cerebral dysfunction characterized by disordered awareness, attention, and cognition." *(337)*

Delirium may be transient and fluctuate over the course of the day and can be classified as hyperactive, hypoactive, or mixed *(338*, *339)*. Understanding the underlying etiology of the delirium is essential for appropriate treatment. Risk factors may include comorbid illness, advanced age, prior dementia, hypoalbuminemia, infection, azotemia, and/or medications *(340–342)*. The prevalence of delirium in patients with cancer ranges from 28 to 48% *(343–345)* and approximately 90% of patients will experience delirium hours before death *(343*, *346*, *347)*. In HCC, delirium and even coma may result from increased levels of ammonia over the course of the disease but particularly at the end of life *(348)*. Appropriate assessment and differential diagnosis is critical in treating cognitive impairment in patients with HCC. Education and support can also be provided to the family caregivers, as they are often the first to recognize the changes in mental status, to facilitate the treatment of the symptoms.

6. SPECIAL ISSUES

6.1. Interpersonal Context of HCC

The patient is often not alone when facing diagnosis and treatment of HCC. The patient's social environment includes family, friends, and work colleagues, all of whom can be affected by the diagnosis and treatment of cancer. The immediate "family" is most likely affected as they often provide immediate care of the patient. Whether the patient is married or cohabitating with a partner, disruption of the relationship is not uncommon. The diagnosis of cancer can increase stress and in a relationship that already is strained, the additional stress associated with the diagnosis and treatment may result in further discord which may be reflected in problems with communication, caregiving, and maintenance of the relationship *(138*, *349–355)*.

An entire literature has been devoted to understanding and ameliorating caregiver stress across chronic diseases and specifically cancer *(356*, *357)*. Increasing evidence has suggested that some family caregivers may experience as much or greater levels of stress than patients who are suffering from a chronic illness such as cancer *(355*, *358–360)*. Several studies have reported that caregivers have increased levels of stress, depression, and decrements in quality of life when compared to persons who are not caregiving *(365*, *359*, *360)*. As a result of the stress associated with caregiving, suppression of immune system functioning *(361–363)* and increased mortality have been reported *(364–366)*.

Psychosocial support of families should be incorporated into overall care of the patients, as family caregivers perform a majority of the caregiving responsibilities today, and this is likely to increase in the coming years *(367)*.

Family caregivers often not only need to care for the patient but often learn new roles and skills they may have not previously possessed. Caregivers may also have their own medical problems and/or stressors in addition to caregiving responsibilities.

Healthcare professionals may facilitate the support of caregivers. Encouraging self-care including diet, exercise, and sleep are critical, particularly if the caregiving extends over a long period of time *(368)*. Requesting assistance from other family members and/or organizations that provide support is recommended. The patient may not initiate discussions regarding their own death or the caregiver's future after their death and caregivers may find it difficult to discuss these issues as they do not want the patient to feel they are giving up hope or wishing they would die. The healthcare professional may want to initiate these discussions with the caregiver if appropriate, individually and possibly together with the patient.

Assisting the caregiver in finding ways to bring closure on their relationship and facilitating the opportunity for the patient to share his/her hopes and dreams and for their loved one to help him/her carry those dreams out after his/her death may enhance the relationship *(369*, *370)*. Some caregivers prepare videos, scrapbooks, or recordings of the person diagnosed with cancer to help them maintain a legacy after their death. Dignity therapy is a novel intervention that is currently being tested in patients at the end of life *(371*, *372)*.

Anticipatory grief, which is similar to the grief loved ones' may experience after the death of their family member, but is experienced before an individual's actual death. Often an individual will experience some of the feelings that accompany the stages or phases of grief that have been extensively studied (e.g., denial, anger) *(373*, *374)*. Each person will grieve differently in regard to the intensity and duration. It is important to note the cultural differences that exist in regard to anticipatory and actual grief reactions as this may influence the outward expression of the grief reaction *(375*, *376)*. The predictors of complicated bereavement have not been extensively studied; mixed results suggest that anticipatory grief may benefit the caregiver in preparation for the patient's death *(377–379)*.

Complicated bereavement was seen in the past as severe depression after the loss of a loved one. More recently it is recognized that complicated bereavement may be characterized as the absence, inhibited, or delayed reaction of grief. Complicated grief can also be conflicted (mixed emotions) or chronic (grief reaction is longer than the cultural norm). These complicated grief reactions can result in major depressive disorders, substance abuse/dependence, and/or PTSD. The most serious consequence of complicated bereavement is suicide. The lack of psychosocial support of caregivers after a patients' death is unrecognized and future research con-

cerning interventions to evaluate and treat complicated bereavement in caregivers is warranted.

6.2. Patients with Children and Adolescents

People diagnosed with cancers that are caring for children or adolescents may have additional challenges associated with the ongoing demands of being a parent while undergoing treatment for their cancer. A child or adolescent's functioning can be impaired as a result of the parent's diagnosis and treatment of cancer. Often difficulties in adjustment are manifested behaviorally and difficulties in school, social withdrawal, or symptoms of oppositional or conduct disorder may be observed. In some cases, children may become anxious, depressed, or experience anticipatory grief in reaction to their parent's diagnosis.

Developmentally appropriate communication of the parent's illness and treatment is essential for adjustment. The American Cancer Society and the National Cancer Institute have excellent resources that provide information regarding communication of the diagnosis of cancer to children and adolescents at different developmental stages *(380*, *381)*. Several local and national organizations provide individual and group therapy for children and adolescents whose parents have been diagnosed with cancer, often free of charge (e.g., Cancer Caring Center, Grilda's Club).

6.3. Cultural, Ethnic, and Religious Factors Affecting the Care of the Patient with HCC

As with all cancer, cultural and/or ethnic background as well as religion are important to recognize in regard to the treatment of HCC. Although the cultural factors cannot be generalized to all persons from a particular ethnic or cultural background due to variations in acculturation *(382*, *383)*, culture and/or religious affiliation is important to recognize in regard to the role beliefs, attitudes, and behaviors may play in the diagnosis and treatment of cancer. For example, family members (particularly those interpreting for their parents) from the Middle East and Asia may not want the patient to know their diagnosis *(384*, *385)*. Some cultures or religions may believe that taking an individual's blood may be construed as taking their "life" or "energy" and therefore may not adhere to recommendations for weekly frequent blood work during treatment. Although the scope of this book does not allow a full discussion of this topic, several authors have provided excellent reviews of cultural and religious factors do not resuscitate (DNR) orders that are important in the diagnosis and treatment of cancer *(364*, *365*, *373–379)*.

6.4. End of Life Issues and Existential/Spiritual Issues

Patients and their family members have varying degrees in which they are ready to accept their diagnosis and eventual death from HCC. For some patients, they are prepared to discuss end of life issues at the time of diagnosis while others are never prepared. Issues related to living wills and DNR orders may be addressed in a matter of fact method at the time of diagnosis or early in the treatment process. At this time, the patient may be able to think more objectively about what s/he wishes for at the end of their life rather than during a crisis as death approaches. It is recommended that these types of questions be addressed early in treatment to prevent unnecessary distress for the patient, family, and healthcare provider later when the patient may experience cognitive impairments and be unable to make decisions or the family caregiver is under strain from caregiving responsibilities and distress secondary to the patient's impending death although it is optimal to integrate palliative care at the diagnosis, it should be noted that not all patients and/or caregivers may be prepared to address these issues until later in the disease process.

The most common clinical problems that arise as a patient's disease progresses are issues related to disability, change of roles, and increased dependence. The process is often rather personal and working with the family caregivers is recommended as these issues often affect the caregiver. The patient may have difficulty discussing these issues with their loved one and some patients may express difficulties with acceptance through increased irritability, sadness, or increased interpersonal conflict.

Spiritual or existential issues also often arise at the time of diagnosis or as the disease progresses. Individuals may experience spiritual growth or decline depending on a number of factors, often pre-existing before the diagnosis of cancer *(150, 386–392)*. Some individuals have an increased sense of closeness to their belief in a "higher power" while others feel anger or resentment *(386)*. It is important to recognize that an individual may have mixed emotions regarding their spiritual or existential beliefs. Utilization of the hospital's chaplain services or referral to the individual's own spiritual leader (e.g., priest, rabbi) is recommended to facilitate the patient's ability to address these issues.

Hospice care is often initiated late in the dying process. It is frequently difficult for the patient and healthcare providers to stop active treatment and essentially give up hope for a cure or controlling the tumor growth. Hospice care in the United States often provides patients with a range of services that provide greater comfort at the end of life with professionals trained specifically to assess and treat psychological and physical symptoms at the end of life. Involvement of the palliative care and hospice teams are strongly recommended in the care of HCC patients which is often dependent on the patient and family.

6.5. Alternative or Complementary Medicine in HCC

A high percentage of patients with chronic liver disease, including hepatocelullar carcinoma, seek out alternative or complementary interventions to treat their disease. Although milk thistle in one of the only herbal supplements which is known for its benefits on liver functioning *(393–397)* no clinical trials in HCC have been published *(397)*. No other herbal supplements have been tested in clinical trials and demonstrated to be efficacious or safe. Nonetheless, it is recommended that clinicians query patients about the use of herbal supplements and remain open to discussing these treatments with patients and caregivers. The inability to openly discuss these issues decreases the opportunities to educate patients and caregivers regarding (1) regulations regarding dose/active ingredients in herbal supplements; (2) a paucity of clinical trials that have been conducted regarding the safety, efficacy, and interactions with other medications; and (3) lack of available information regarding metabolism of the drugs in the liver, particularly the cirrhotic liver. Encouraging dialogue and providing further information (e.g., National Institute of Health's Institute on Complementary and Alternative Medicine) is recommended as criticism or lack of discussion will likely result in continued use without the disclosure to the medical team. It is increasingly being recognized that some of the herbal supplements may up- or down regulate chemotherapeutic agents (e.g., sorafenib), therefore the importance of documentation of the patients' prescribed medications as well as supplements is critical for optimal response to treatment.

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25 Putting It All Together

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CONTENTS

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In the previous chapters there has been a systematic description of HCC as a disease, its etiology, clinical presentations, the various diagnostic tools, and treatment options that are available. The intent of this chapter is to try to offer some practical guidelines for the physician seeing a patient for the first time and some considerations of common management choices.

1. SCREENING FOR HCC

Much has been written on the subject of screening for HCC, including the usefulness of α-fetoprotein as a marker and the best, simplest, and cheapest radiologic modality. There have been several papers showing that the cost–benefit of screening has not been proven, as judged by the cost for screening large populations that are known to be at risk compared to the small numbers of tumors that are detected at a treatable stage, as well as the false-positive outcomes. Without prejudice to the outcome of this ongoing debate, a patient in the United States that has chronic HBV, chronic HCV, or is known to be cirrhotic from any cause, is at risk for subsequent development of HCC. Cirrhosis is thus a premalignant condition. Considering that we know the cause of so few cancers of adult humans, it seems to us that the physician has an obligation to follow up on patients with these diseases, who are known to be at risk, in the hope of early diagnosis and therefore finding the HCC at a treatable stage. It is our practice therefore to do twice yearly CT scans and α-fetoprotein measurements, even though the latter are elevated in only 50%

of HCCs and there is no clear linearity between tumor size and α-fetoprotein measurement. Given that the published figures for development of HCC in a patient with cirrhosis are between 2 and 5% per annum, it might be expected that routine annual or semi-annual screening of patients with cirrhosis is likely to detect a reasonable number of HCCs at a treatable stage. All this needs to be weighed against the cost of managing patients at advanced stage at diagnosis.

2. THE ROLE OF BIOPSY

Fine needle aspiration biopsy (FNA) is a well-established routine and can detect cancer. It normally cannot supply the architecture for a confident diagnosis of HCC. Usually, only core needle biopsy can do that. Recent practice in some parts, particularly in Europe, is to avoid biopsy when there is presence of cirrhosis, a vascular liver lesion, and a rising α-fetoprotein level. It is our practice always to do a biopsy before treatment, whenever practical. We believe that this is important, since it gives us complete confidence that we have the correct diagnosis and the correct tumor histological type; and second, as we enter the age of molecular proteomics and molecular diagnostics, there are an increasing number of tests that are starting to permit us prognostic group stratifications that require tissue for either special stains, in situ hybridization, or gene expression. It has been argued that percutaneous needle biopsy is associated with a risk of spread by needle tracking. Although this has been reported, in our experience of 1,300 needle biopsies for confirmation for the presence of HCC, we have seen this only in seven cases, and all of them have been in the track of the needle, typically the chest wall, and therefore easily treated. As with everything in medicine, there is a risk–reward calculation that needs to be made. We believe that the benefit or reward of getting a correct tissue diagnosis and tissue for prognostication hugely outweighs the very low risk of needle tracking, an even rarer risk of tumor bleed or other rarer complications associated with the presence of ascites.

3. WHAT IF THE FIRST BIOPSY COMES BACK NEGATIVE FOR CANCER OR IS INCONCLUSIVE?

There are several choices in this situation. These include a repeat biopsy, laparoscopic biopsy, or repeat CT scan and then biopsy in 3–4 months time, especially if any one of the tumors appears to be growing. Sometimes there can be multiple less than 1-cm nodules and two or more biopsies proven to be negative. This can be a difficult situation, and repeat CT scan follow-up is clearly indicated in this situation.

4. METASTATIC DISEASE INVOLVING THE LUNGS, BONES, OR BRAIN

Symptomatic approach is required for all cancers including brain radiation for brain metastases and spinal radiation for lytic or blastic metastases that put any spinal vertebra or the pelvis at risk. The literature really does not support any chemotherapeutic agent or combination of agents as being effective in this situation. We put all our patients on phase II or phase I studies for extrahepatic metastases. However, we often find patients whose disease is almost entirely confined to the liver other than some periportal lymphadenopathy. In this situation, we focus on the 99% of the disease that is in the liver and simply watch the lymph node disease. Quite often, this never seems to change. If it does enlarge, however, it can normally be dealt with using external beam ionizing radiation.

5. WHAT IS THE BEST TREATMENT FOR ONE TO TWO HEPATIC LESIONS, EACH 3 CM OR LESS?

The choices here depend upon the location and proximity to major vessels or bile ducts but usually consist of PEI, RFA, or TACE. If the lesions are accessible, then either PEI or RFA, depending upon the operator skill and interest, would seem to be equivalent, and for small lesions at least, resection appears to be equal to PEI. The choice of treatment is also impacted by the severity of cirrhosis. Additionally, given the favorable curative new MELD criteria, liver transplant is a reasonable treatment option in this situation, especially in the presence of cirrhosis.

We have a multidisciplinary weekly Liver Tumor Conference, where all new and difficult cases are reviewed, prior to a treatment decision.

6. WHAT ARE THE TREATMENT OPTIONS FOR ONE TO TWO LESIONS OF ANY SIZE, WITHOUT CIRRHOSIS OR WITH CIRRHOSIS BUT NORMAL LIVER FUNCTION TESTS?

A single lesion of any size in a noncirrhotic liver, or Child A cirrhosis, and a small contralateral lesion. Depending on the exact location and proximity to major blood vessels, resection of both lesions or resection of one lesion with RFA of the contralateral lesion might be a reasonable choice. If the main lesion cannot be resected, then TACE or hepatic ^{90}Yttrium microspheres would be our preference. If cirrhosis is present, liver transplant should be considered, given the favorable MELD score and the chance for cure.

7. WHO SHOULD OR CAN RECEIVE A LIVER TRANSPLANT?

Anyone can receive a transplant for HCC; however, only certain patients can receive an upgrade to their UNOS waiting status (i.e., shorten the waiting time to transplant). The current UNOS guidelines include HCC as a single lesion less than 5 cm maximum diameter or three HCC lesions each equal to or less than 3 cm without gross vascular invasion of a main portal vein or a portal vein branch or a hepatic vein branch and without metastases, regardless of the degree of cirrhosis. These patients have the highest possibility of complete cure since the liver transplant treats both the cirrhosis and the HCC, unlike the above treatments. The UNOS (cadaveric) and MELD scoring systems are regularly updated.

MELD (Model for End-stage Liver Disease) was instituted on February 27, 2002, with a 6- to 40-point scale based on serum total bilirubin, INR, and creatinine, with more severe disease having a higher score (http://www.unos.org/resources/meldpeldcalculator.asp). For patients with radiographic evidence of stage I HCC (1 tumor up to 2 cm), 24 MELD points were assigned, and for those with stage II HCC (1 tumor up to 5 cm or up to 3 lesions all less than 3 cm, without gross vascular invasion or extrahepatic spread), 29 points were assigned. After 1 year, it became evident that this was too high a priority, and the points were deleted for stage I HCC and decreased to 22 for stage II.

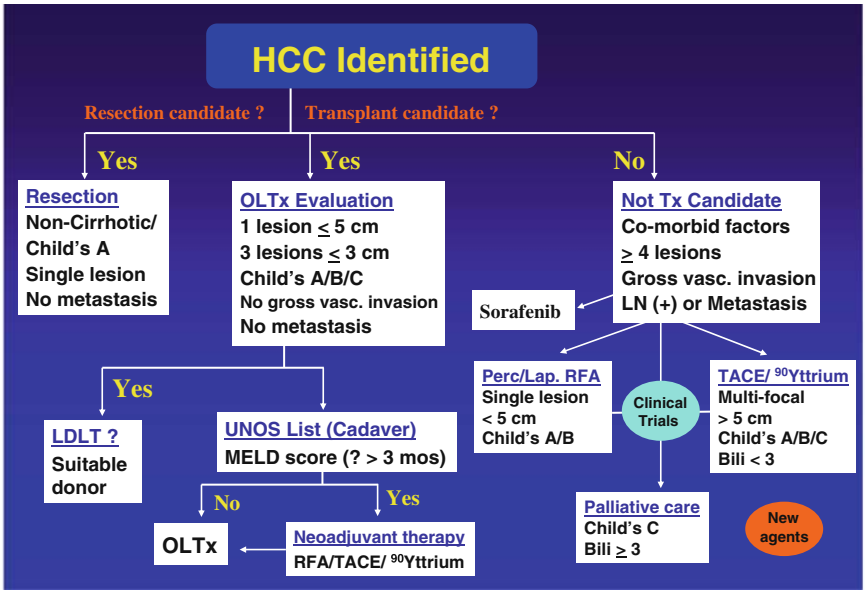

Table 6

8. WHAT ARE THE TREATMENT OPTIONS FOR ONE LESION GREATER THAN 5 CM OR THREE LESIONS WITH ONE OR MORE GREATER THAN 3 CM?

We approach this with TACE or hepatic ^{90}Yttrium in an attempt to downstage the size or the lesion in question. Once the patient has been restaged and can fit within the MELD score criteria for transplant, then the patient has a liver transplant evaluation and is listed, if appropriate though they will not receive upgrade points on the UNOS waiting list since their initial stage was >2. Alternatively, the patient can be transplanted as a primary treatment (depending upon the philosophy of the individual transplant center), but the patient will not receive any additional MELD listing points.

9. A PATIENT WITH MULTIPLE LESIONS, ANY GREATER THAN 5 CM AND WITHOUT METASTASES, WHO HAS A BLOOD GROUP/MATCHED FAMILY MEMBER WILLING TO ACT AS A LIVING-RELATED DONOR

Live donor transplantation has been used frequently in the past for patients with HCC due to the shortage of organs and rapidity of HCC growth. However, with the recent advent of the allowance of extra MELD listing points for patients with HCC (single lesion $\leq$5 cm or three lesions none >3 cm), the incidence of live donor transplants for this group of patients has decreased. For those patients with single lesions >5 cm or more than three lesions, live donor transplantation is an option but is individualized within each transplant program. Because the risk of recurrence in this group of patients is much higher, many programs will not offer live donor transplants to this group. However, as we have recently found, patients with multiple lesions may have either multiple de novo tumors or intrahepatic metastases; these groups can be distinguished using currently available genotyping techniques. Patients with multiple small de novo could be considered for live donor transplantation while the recurrence rate for patients with intrahepatic metastases is usually prohibitive. If the patient has a single, peripheral lesion greater than 5 cm without metastasis or hepatic/portal vein involvement, the patient could be considered for live donor transplantation.

10. MULTIFOCAL HCC WITH TUMORS CONFINED TO THE LIVER WITH OR WITHOUT PORTAL VEIN THROMBOSIS AND BILIRUBIN LESS THAN 2.0 MG/DL

These patients are treated with hepatic artery chemotherapy or chemoembolization (TACE) or ^{90}Yttrium glass microspheres into the hepatic artery.

Patients seem to prefer the latter because of the minimal side effects and the small number of treatments ever that are usually required.

11. A PATIENT WITH ANY TUMOR, NOT FOR TRANSPLANT, WITH CHILD B OR C CIRRHOSIS, ENCEPHALOPATHY, OR BILIRUBIN GREATER THAN 3.0 MG/DL

These patients are normally referred for palliative or supportive care or possibly phase II studies with noncytotoxic drugs such as hormones or growth factor modulators.

12. CLINICAL EVALUATION AND WORKUP FOR LIVER TRANSPLANT

The patients are evaluated by a multidisciplinary team at most transplant centers consisting of transplant surgeons, hepatologists, anesthesiologists, nurses, and social workers. The evaluation includes a thorough history and physical exam as well as an evaluation of his/her cardiac and pulmonary functions. All patients have an endoscopy to assess for esophageal varices. Further, age-appropriate screening for other carcinomas should be performed (e.g., colonoscopy. mammography, pap smears). Blood work for tissue typing, tumor markers, viral disease (e.g., HBV, HCV, HIV, CMV, EBV), and autoimmune markers is done. All patients with HCC being considered for transplantation must have a current CT/MRI of the abdomen and pelvis as well as a CT of the chest. After the medical testing and fiscal clearance are obtained, the patient is presented at the Transplant Evaluation Conference for listing.

13. NEEDED CLINICAL TRIALS

In addition to the clinical trials that are needed and mentioned at the end of Chapter 2 , certain randomized controlled clinical trials (RCTs) and their results are needed for our subject to move forward, based on the evidence that only randomized clinical trials can provide. Some of the more pressing problems are the following:

a. For nonsurgical tumors, can the benefits of tumor shrinkage with intrahepatic arterial TACE or ^{90}Yttrium microspheres on the one hand and the small survival advantage with oral kinase/angiogenesis inhibitors such as sorafenib or erlotinib plus bevacizumab on the other hand be enhanced by combining these two modalities?

b. Can the high recurrence rates after surgical resection be reduced, and life extended, by combining resection with either neoadjuvant (prior) or adjuvant (after) resection therapy? Candidates for RCTs include the kinase/angiogenesis inhibitors or ^{90}Yttrium microspheres (a single trial showed benefit for ^{131}I-lipiodol, but multiple trials showed no benefit for chemotherapy).
c. Liver transplant for Milan-extended criteria. Transplants for T1 and T2 HCC lesions have similar survival as patients who are transplanted without cancer. It is clear that liver transplantationcadaveric or live donorcan enhance the survival of patients with liver failure and more advanced HCCs, but the results are not as good as for smaller tumors. In order to determine whether the limits of transplantation for advanced HCC can be extended, trials are needed for adjuvant and neoadjuvant medical therapies in the transplant setting, just as for resection. So far, there have been none.
d. Identifying patient subsets in differing prognostic bands. It has been known for a long time that the limits of classical pathology have long been reached, in identifying patients who are more or less likely to have prolonged survival, within identical staging parameters. Even the early papers on liver transplantation of Pichlmayer and Iwatsuki *(1, 2)* showed a tail of 20% of patients with advanced HCC stages III and IVa tumors who had long survival. With the increasing reports of the identification of gene expression patterns or specific genes that correlate with better or worse survival, the era of molecular classification has arrived and the new molecular markers will need to be validated in clinical trials to determine their usefulness. These will be necessary for patient stratification both in surgery and especially for medical treatment. If the multikinase inhibitor sorafenib really targets Raf and VEGFR as described, then it is likely that future patient selection will depend on those whose tumors express these target proteins. Similarly with erlotinib and EGFR, and bevacizumab and VEGF-A.

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