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Brian I. Carr
Editor

Hepatocellular Carcinoma

Diagnosis and Treatment

Third Edition

Springer

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Maurie Markman

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To my daughters, Ophira and Feridey
And to their sons, Rohan, Kunal and Oren

He used to say:
If I am not for myself, who will be for me?
And if I am only for myself, what am I?
And if not now, when?

—Hillel, Mishna Avot 1:14

Who is wise?
He who learns from every person.

—Ben Zoma, Mishna Avot 4:1

What we see changes what we know. What we know changes what we see.

—Jean Piaget

In questions of science, the authority of a thousand is not worth the humble reasoning of a single individual.

—Galileo Galilei

Preface to the Third Edition

In the interval between the second edition of this book in 2009 and this new, third edition, there have been immense advances in both the science and the clinical practice of hepatocellular carcinoma (HCC). The advances are already being built upon to enlarge our understanding of this complex and heterogeneous disease, which is increasing in some parts of the world and decreasing in others. As a result, the original chapters have been updated and more than a dozen new chapters were added, on the following topics: molecular profiling, molecular mechanisms in hepatocarcinogenesis, genomic phenotypes, miRNAs, gene signatures of risk factors, gut microbiota, microenvironment, tumor heterogeneity, circulating tumor cells, immune system and therapy, inflammation, obesity and NASH, staging systems, CT and bioenergetics. Many of the previous chapters have been completely rewritten, including those on local ablation, resection, transplantation, and the final summary chapter. The general scope of these advances is as follows:

1. The introduction into clinical practice of FDA-approved and effective drugs for HCV, with sustained virological responses obtainable for both HBV and HCV, together with high cure rates for HCV.
2. Initial clinical studies showing that the high tumor recurrence rates postresection can be reduced, not by anti-tumor therapy but, by treating underlying virus hepatitis. If confirmed, they will have major conceptual implications for our ideas about HCC therapy and antivirus therapy will be viewed as part of HCC therapy.
3. The underlying cirrhosis (non-HCC part of the liver) is increasingly being seen as not just a comorbid disease (although it is), but also as a source of prognostic information and determinant of HCC biology. Like items #1 and 2, it indicates that the microenvironment is a source of many HCC influences, including immunological, inflammatory, neovascular, cytokine and growth factor actions.
4. Systemic inflammation has become an important and independent prognosticator for many tumor types, including HCC and the simple 2-parameter Glasgow score and its variations are incorporated into clinical practice.
5. Molecular profiling is being used to identify HCC phenotypes, lineage subsets and hopefully, will support rational therapy selection (for example, Met-expressing tumors for Met inhibitor therapies). Furthermore, the increasing commercial availability of kits for purifying tumor cells or free tumor DNA in the blood circulation may provide a safe way of obtaining specific HCC information without the hazards of biopsy, as well as an easy and safe way to provide samples for molecular profiling at various phases of the HCC clinical course in the same patient.
6. Immune checkpoint inhibitors are taking center stage for therapy of many cancer types, with promising early results in HCC.
7. Extended criteria for transplanting larger HCCs and identification of prognostic subtypes are gaining traction.
8. ^{90}Yttrium microspheres regional therapy is being recognized as a safer alternative to TACE in the presence of portal vein invasion.

9. Several large phase III trials of new non-sorafenib (multi-)kinase inhibitors failed to meet their expected goals. However, many new targeted agents are currently being evaluated in clinical trials. Furthermore, trials are in progress that examine the combinations of either targeted therapies such as sorafenib with regional therapies (TACE or ^{90}Yttrium microspheres), or two or more therapies that target different pathways. In addition, ways of enhancing sorafenib effects or decreasing resistance to its actions are under investigation.
10. We are seeing the development of drugs against new, nongrowth signaling targets, including putative tumor stem cells, dendritic cells, tumor invasiveness proteins, growth-antagonizing microRNAs; the development of tumor vaccines and novel nuclides for internal radiation, such as ^{166}Holmium and ^{188}Rhenium, intensity modulated radiation and proton beam therapy.
11. There is a considerable increase in obesity-associated HCC and its different pathogenesis from virus-mediated HCC. This may supplant hepatitis as a cause of HCC in the Western world. The interplay of several factors in many HCC patients, such as HBV and alcohol, HBV and aflatoxin B_1 dietary exposure.
12. There has been a proliferation of proposed staging systems from several countries. Some systems are seemingly more applicable to patients in certain regions of the world than other systems.
13. The sorafenib phase III SHARP trial highlighted the discrepancy between tumor responses and patient survival, as shown by the minimal number of partial objective tumor responses (tumor size change) on the one hand and the finding of significant sorafenib survival benefits on the other. This has consequences for our thinking about the relevance of tumor size change in HCC (especially mediated by cytotoxic chemotherapy) and how we assess useful clinical endpoints for future HCC therapy trials. One result is a reconsideration of the value of 'stable disease' as a desirable endpoint in HCC management.
14. The pace of discovery is quickening, as is the interplay of the basic science and clinical applications. Perhaps the most profound changes have resulted from the availability of an effective vaccine against HBV or primary prevention (though not yet against HCV), and the new effective treatments for both HBV (non-curative) and HCV (curative). Thus, primary, secondary, and tertiary prevention are now available: primary prevention, by vaccination (HBV only); secondary prevention, by treatment of chronic carriers and decreasing the probability of developing cirrhosis and subsequent HCC; and tertiary prevention, by anti-hepatitis therapy resulting in the suppression or eradication of the hepatitis infection, with resultant decreases in postresection HCC recurrences.

Thus, the most significant recent translational advance has been in the area of hepatitis prevention (HBV) and treatment (HBV and HCV), with profound effects on the incidence and likely the biology of HCC caused by hepatitis B or C.

The book is divided into three parts: I, Causes, Biological and molecular basis; II, Diagnosis; III, Therapies. The final chapter provides an overview of current therapy.

November 2015 Brian I. Carr

Preface to the Second 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 multihead 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, nonmetastatic HCC seems to bestow a survival advantage compared with 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 with added chemotherapy or ^{131}I lipiodol. The introduction of ^{90}Y microspheres (Theraspheres) appears to offer the promise of relatively nontoxic 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, as 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 the First 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.

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.

Brian I. Carr, MD, FRCP, Ph.D.

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Part I
Causes, Biological and Molecular Bases of HCC

Epidemiology of Hepatocellular Carcinoma

1

Donna L. White, Fasiha Kanwal, Li Jiao, and Hashem B. El-Serag

Contents

1.1 Global Incidence of Hepatocellular Carcinoma

1.1.1 Overview

Primary liver cancer or hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, with liver cancer accounting for 9.1 % of global cancer mortality [1]. In 2012, there were an estimated 782,000 incident HCC cases. Given an almost equally high number of deaths, 746,000, the mortality-to-incidence ratio is 0.95. Across time periods, regions and genders, liver cancer typically occurs in middle-aged and older adults. However, the burden of HCC is not evenly distributed throughout the world (Fig. 1.1). It also disproportionately impacts males (Fig. 1.2), with HCC the second leading cause of cancer mortality in men and the ninth leading cause in women [2].

Globally, the vast majority of HCC cases occur (>83 %) in less developed regions, particularly in Eastern and South-Eastern Asia and sub-Saharan Africa. China alone account for 50 % of all HCC cases, with an age-standardized incidence rate (ASR) of 22.3/100,000 person-years in 2012 [1]. However, four other countries have even higher ASRs—Mongolia (78.1/100,000), Lao People's Democratic Republic or Laos (52.6/100,000), The Gambia (25.8/100,000), and Egypt (25.6/100,000). Some typical rates from medium rate countries (i.e., HCC ASRs between 5 and 20/100,000) include Italy (7.6/100,000) and Spain (5.9/100,000). Notably, increasing numbers of countries that were formerly low rate (ASRs < 5/100,000) even a decade ago have now become medium rate countries, including the U.S. which had an ASR of 6.1/100,000 in 2012 (Fig. 1.3). Some rates from typical low rate countries include those from Argentina with an ASR of 3.3/100,000 and Israel with an ASR of 2.3 [1].

HCC accounts for between 85 and 90 % of primary liver cancers in adults [3]. One noteworthy exception is the Khon Kaen region of Thailand, which has one of the world's highest rates of liver cancer. However, due to endemic population infection with liver flukes, the major type of liver

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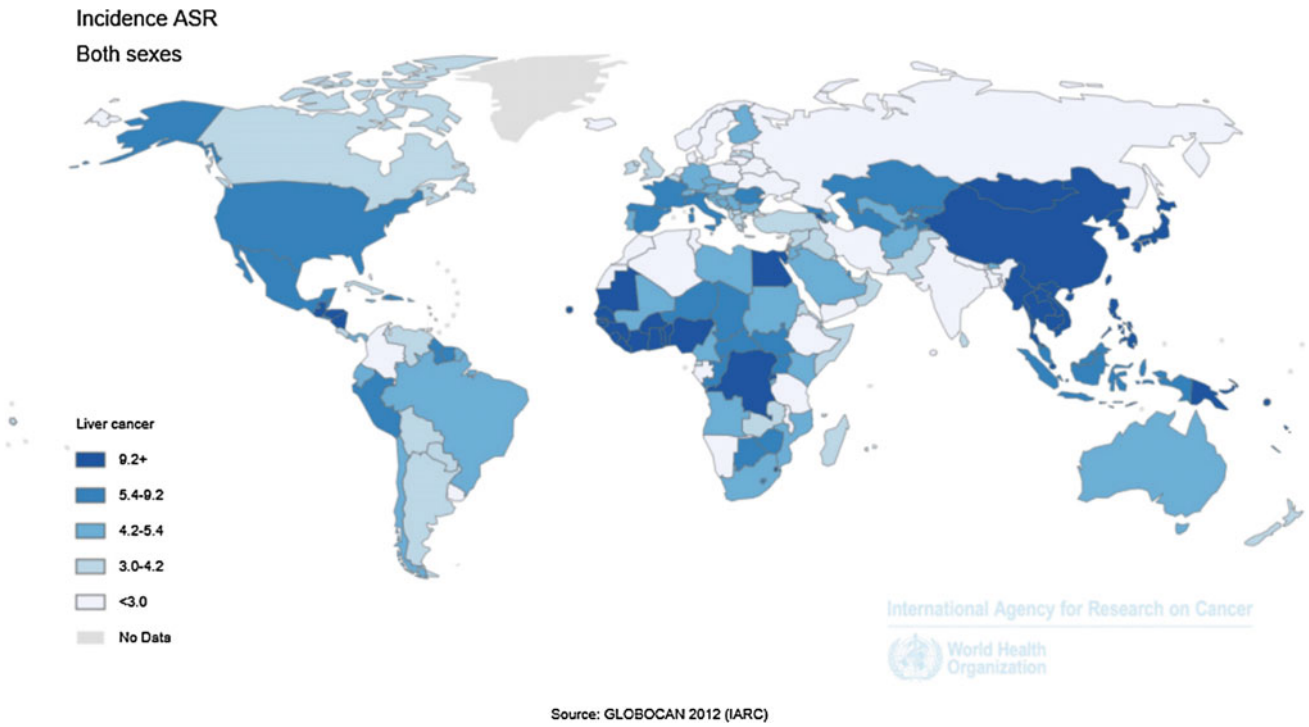

Fig. 1.1 Global map of age-standardized rates (ASR) of HCC in 2012

cancer in this region has historically been intrahepatic cholangiocarcinoma (ICCA) rather than HCC [4]. Mass drug administration and public health education campaigns that began in the early 1980s have resulted in a dramatic decreased prevalence of liver fluke infection in the population from 80 to 15 to 20 % by 1997 and remaining stable through 2013 [5]. This has led to subsequent substantial declines in ASRs for ICCA in this region with ASRs of 67.6/100,000 in males and 27.3/100,000 in females for the period 2004–2006, [6] levels already showing reductions from reported historical levels ranging between of 85–90/100,000 in males and between 32–39/100,000 in females [5]. Overall, ICCA remains the second most common primary hepatic malignancy worldwide, with over 750,000 million people residing in areas endemic for liver flukes and thus at ongoing exposure risk (e.g., Poland, Germany, Russia, Kazakhstan, and Western Siberia for *O. felineus*; Korea, China, Taiwan, and Vietnam for *C. Sinensis*, and North East Thailand, Laos, Cambodia for *O. Viverrini*) [7, 8] with an estimated 56.2 million persons globally infected with foodborne trematodes in 2005 [9].

Overall encouraging trends in HCC incidence have been seen in some high-rate areas. For example, between 1978 and 1982 and 1993 and 1997, decreases in incidence were reported among Chinese populations in Hong Kong, Shanghai, and Singapore [3] (Fig. 1.3). These rates continue to decline (e.g., China/Hong Kong ASR: 23.6/100,000 in 2001 vs. 18.9/100,000 in 2011) [1]. In addition to these areas, Japan also began to experience declines in incidence rates among males for the first time between 1993 and 1997.

Many high-rate Asian countries now vaccinate almost all newborns against hepatitis B virus (HBV) and the effect on HCC rates has already become apparent. In Taiwan, where government mandated national newborn vaccination began in 1984, HCC rates among children aged 6–14 years declined significantly over a short period from ASR: 0.70/100,000 in 1981–1986 to ASR: 0.36/100,000 in 1990–1994, [10] an effect that was presumed to be to vaccination. However, a national cohort analysis to evaluate the relative importance of age, time trend (period), and vaccination (cohort) on HCC incidence and mortality in Taiwanese children suggests HCC rates were already notably declining in boys and especially in girls in the 1980s prior to HBV vaccination, and that the first substantial vaccine-related decline in HCC rates was seen in boys starting 2000–2004 (i.e., a 15 year time lag) [11]. It is too soon yet for HBV vaccination to have had a substantial effect on adult rates which are highest in middle-aged and older adults, but other public health measures have likely 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 reduced exposure to known hepatocarcinogen aflatoxin B1 (AFB1) in this area [12]. 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

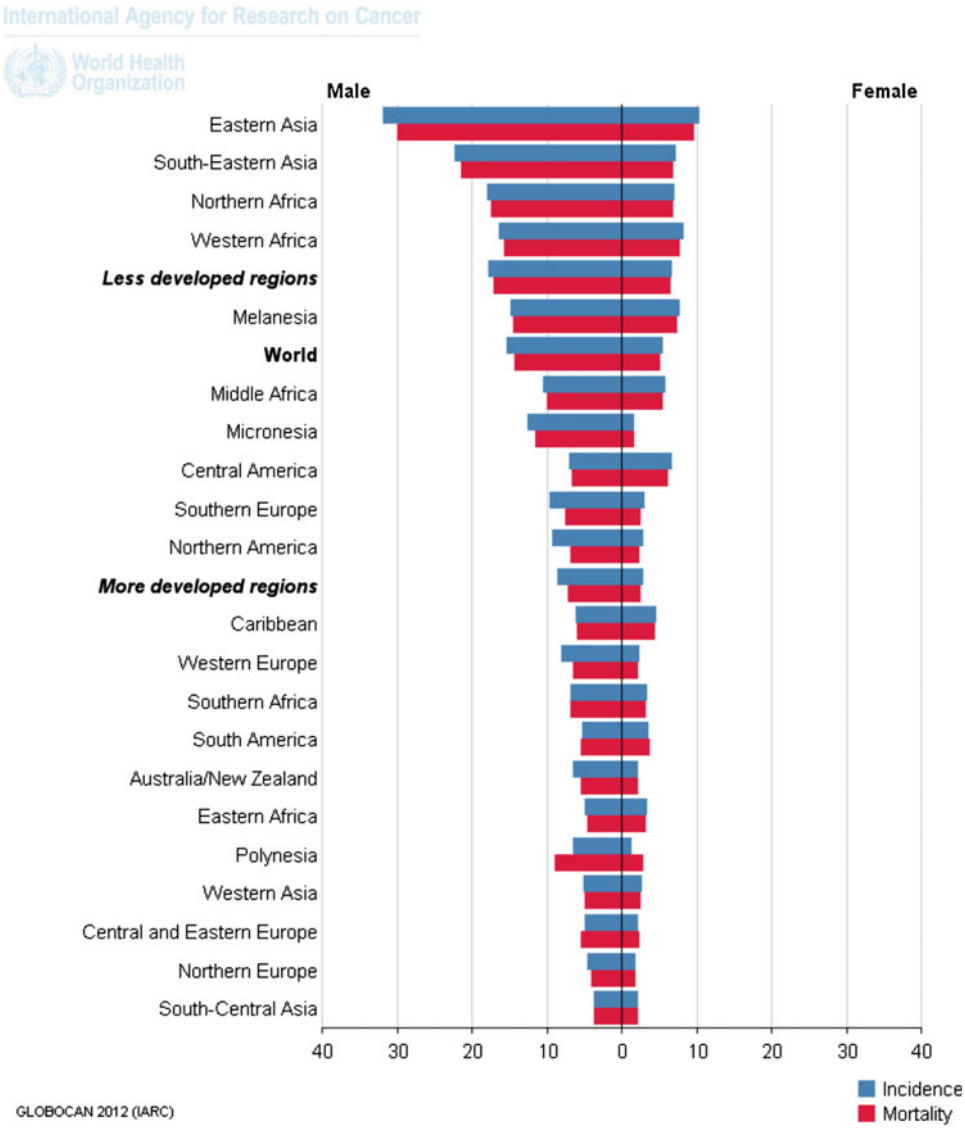

Fig. 1.2 Gender-specific and age-standardized HCC incidence and mortality rate by region and development status in 2012

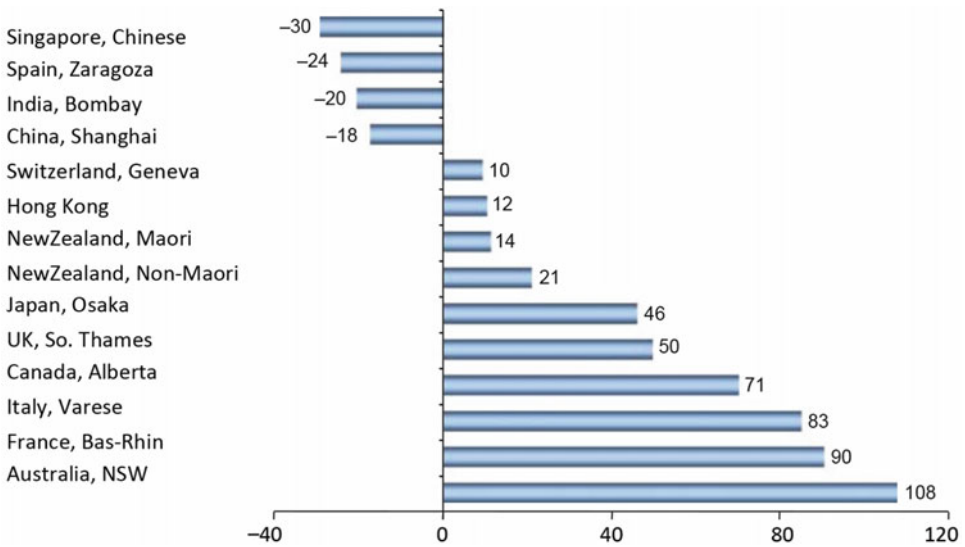

Fig. 1.3 Recent changes in the incidence of HCC. The incidence of HCC has been declining in some "high incidence" areas, such as China and Hong Kong. On the other hand, HCC incidence in several "low and intermediate incidence" areas has been increasing. Modified from McGlynn et al

decreased consumption of microcystins, blue-green algae (cyanobacteria) produced compounds demonstrated to be hepatocarcinogenic in experimental models [13].

In contrast, registries in a number of low- and medium rate areas reported sizable and continuing increases in HCC incidence between 1978–1982 and 1993–1997 [14] (Fig. 1.3). Included among these registries are those in the United States, the United Kingdom, and Australia. Reasons for both the decreased incidence in historically high-rate areas and the greatly increased incidence in formerly lower rate areas are not completely understood, suggesting that each will be an important case study. It has, however, been widely hypothesized that most of the increased incidence in many lower rate areas with ongoing low rates of HBV infection is due to the rapid aging of their hepatitis C virus (HCV) cohorts combined with substantial increasing rates of obesity and diabetes over the last few decades.

1.1.2 Race/Ethnicity

HCC incidence rates can vary greatly among different populations living within the same region. For example, in the United States at all ages and among both genders, age-standardized rates (ASRs) of liver cancer, expressed, per 100,000 are higher in Asians and Pacific Islanders (13.1) than in Hispanics (11.0), African-Americans (8.0), or Whites (4.5) [15]. The reason(s) for this interethnic variability likely include differences in prevalence and time of exposure for major risk factors for liver disease as well as for HCC, and potentially, in prevalence of salient genetic polymorphisms (e.g., the much lower prevalence among individuals of African ancestry of the highly favorable *IL28B* allele for HCV). Interestingly, liver cancer rates can also vary considerably individuals of the same race/ethnic group living across large geographical expanses, e.g., ASRs (expressed per 100,000) for Chinese males residing in China (Bejing): 16.7; Hong Kong: 26.7; Malyasia (Penang): 10.5; U.S. (Los Angeles): 18.4 [16] or even within the same country (e.g., very high ASRs in Mekong area including Khon Kaen in northern Thailand vs. considerably lower rates in Bangkok in southern Thailand). The reasons for this intra-ethnic variability likely include differences in exposure to and/or acquisition time of other liver disease risk factors (e.g., liver flukes, dietary aflatoxins, obesity, and alcohol use). However, they may also be impacted by relative differences in underlying population structure (e.g., age and gender) as well.

1.1.3 Gender

In essentially all populations, males have higher reported HCC rates than females, with male-to-female ratios usually averaging between 2:1 and 4:1 (Figure 1.2). As of 2012, some of the largest discrepancies in rates (>3.5:1) are found in medium rate European populations. Typical among these ratios are those reported in registries in Volume *X* of Cancer in Five Continents: Biela, Italy (3.9:1); Munich, Germany (3.6:1); Geneva, Switzerland (4.4:1) [17]. Among the 11 French registries, nine reported male:female ratios > 5:1. The gender ratio in the U.S., which is also medium risk, is lower (3.3:1). In contrast, typical gender ratios currently seen in high-rate populations are generally lower and include those of Qidong, China (3.0:1); Osaka, Japan (2.9:1); and Harare, Zimbabwe (1.2:1). Registries in Central and South America report some of the lowest sex ratios for liver cancer. Typical ratios in these regions are reported by Pasto, Colombia (1.2:1), and Costa Rica (1.6:1).

The reasons for higher rates of HCC 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 visceral adiposity. Yet there are several compelling reasons to believe that sex-based biological differences (e.g., genetic, sex hormone levels) may also contribute to this pervasive dimorphism: dimorphism persists after adjustment for gender-based differences in other known risk factors, is observed in human children, and is evident in animal models. Further, use of some sex hormone modifying medications including androgenic anabolic steroids and earlier high dose formulations of oral contraception have been associated with young onset HCC in some case reports. However, the role that normal variation in sex hormone signaling plays in the substantial unexplained interindividual variability among individuals of the same gender and with similar major risk factors for HCC is not known.

The global age distribution of liver cancer varies by region, incidence rate, gender and, possibly, by etiology [18]. HCC overwhelmingly occurs in adulthood, most often as HCC arising in the background of one or more environmental or behavioral exposures known to increase liver cancer risk. Although the overwhelming majority of HCC are sporadic or have no similarly affected first-degree relative, family clusters [19] and also significant additional increases in HCC risk even after accounting for hepatitis status have been reported [20]. In contrast, the most common liver cancer in children is hepatoblastoma (∼2–3 cases/per

million persons) that arises in the background of a genetically determined disorder like Beckwith–Wiedemann syndrome, glycogen storage disease type I, or Tyrosinemia in infancy thru early childhood (most occurring within the first 18 months).

In adults, female HCC rates typically peak in the age group 5 years older than the peak age observed in comparable males. In low- and medium-risk populations (e.g., Canada, the United Kingdom, and the United States), the highest age-specific rates occur among persons aged 75 and older [18]. A similar pattern is seen among most high-risk Asian populations (e.g., Hong Kong, Shanghai). In contrast, male HCC 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 become infected as adults, most HBV carriers become infected at very young ages.

A historical exception was Qidong, China, where HCC rates are among the world's highest and where age-specific incidence rates among males rose until age 45 and then plateaued, while among females, rose until age 60 and then plateaued. The reasons for this unusually early onset are unclear, but could be due to existence of other hepatocarcinogenic exposures or differences in dose and timing of known hepatocarcinogens like dietary aflatoxin. However, by 2005–2008 the age of onset for the first time had increased to over 50 years, an effect hypothesized to be largely attributable to public health prevention measures in the region particularly dietary shift to prevent aflatoxin exposure [12].

1.1.4 HCC in the United States

Research conducted using the National Cancer Institute's (NCI) population-based Surveillance Epidemiology and End Results (SEER) registry data which cover >13 % of the U.S. population showed that overall annual age-adjusted HCC incidence rates (per 100,000) doubled from 1.4 in 1975–1977 to 4.8 in 2005–2007 [18] (Fig. 1.4) with large increases in incidence observed among Hispanics and the overall population aged 50–60 years old [21]. Rates continue to rise though not as dramatically, with an annual ~4 % increase in overall incidence observed between 2003 and 2012. This dramatic increase in rates is likely attributable to several factors including rising incidence of cirrhosis particularly due to HCV [22]; substantial recent increases in rates of obesity and thus metabolic syndrome associated

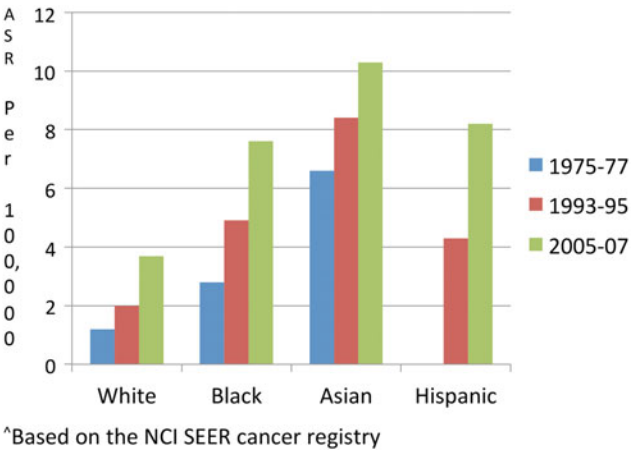

Fig. 1.4 Differences in age-standardized HCC rates by race/ethnicity in the U.S. by time period

complications like NAFLD and diabetes; population aging particularly among the HCV-infected; and a general improvement in survival among cirrhosis patients.

Overall, between 15 and 50 % of cirrhosis and HCC patients in the United States do not have a historically established risk factor like viral or autoimmune hepatitis, a genetic disorder, or an alcohol use disorder [14]. Most of these cryptogenic cases do, however, have some metabolic syndrome features like diabetes or obesity, and thus its hepatic manifestation, nonalcoholic fatty liver disease (NAFLD), is usually the presumptive underlying risk factor [23].

The overall epidemiological profile of incident HCC in the U.S. in 2012 based upon nationwide United States Cancer Statistics (USCS) registry data demonstrated: 73 % of all cases are male; 61 % are White (non-Hispanic); the 55–59 year old age-group has the largest number of incident HCC diagnoses (20 % of total); 89 % of cases are diagnosed at ages 50 and older; and the highest overall age-standardized incident rates (ASR), expressed per 100,000, are found in males who are Hispanic (17.8) closely followed by males who are Asian or Pacific Islanders (17.7). The burden of HCC in the U.S. is also not uniform, with most states with HCC rates in the upper quartile located in southwestern and western regions (Fig. 1.5). In 2012, Texas and Hawaii both reported the highest ASRs 13.7 [15]. Over the last decade, the largest age-specific increases in incidence have been in the 55–59 and 60–64 year old age groups (Fig. 1.6). The NCI's SEER data-based projections for the U.S. in 2015 are that 35,660 individuals in an incident diagnosis in 2015 at a median age of 63 years old, with current estimated overall 5-year survival based upon data from 2005 to 2011 of 17.2 % [24].

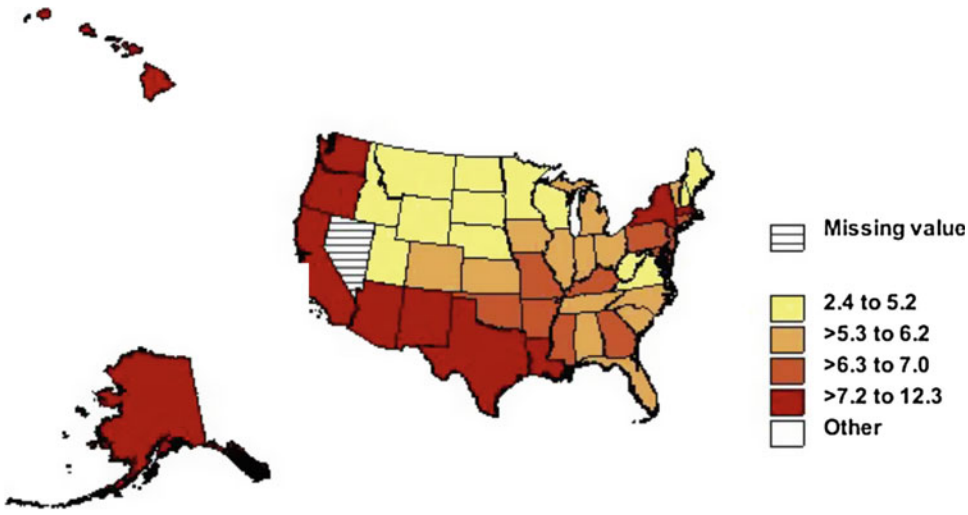

Fig. 1.5 Geographic distribution of age-standardized HCC rates in the U.S. in 2012

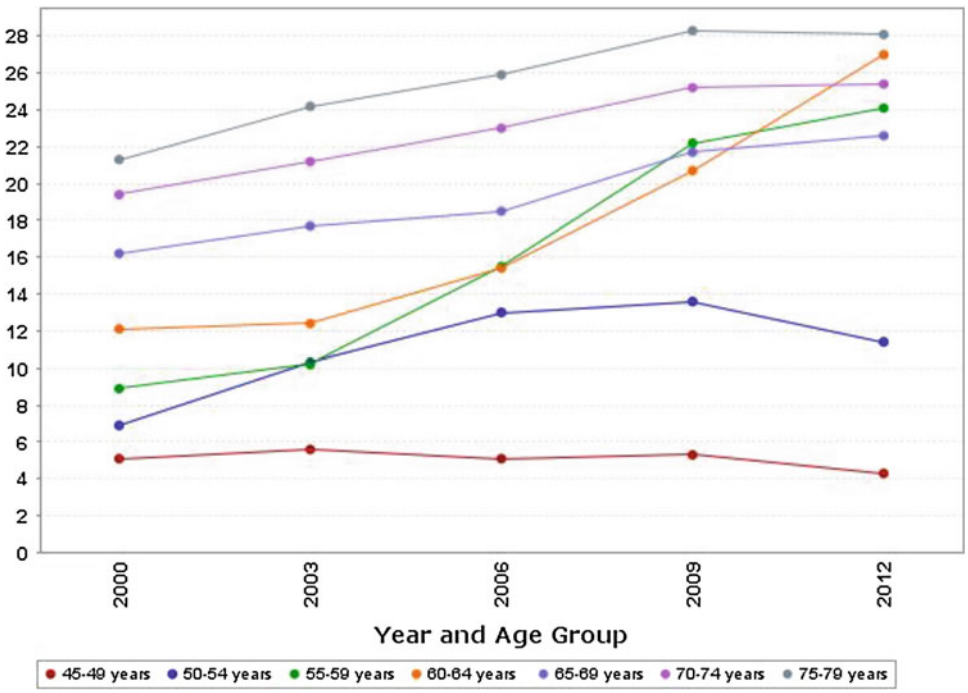

Fig. 1.6 Age-specific incidence rate of U.S. cancer registry reported HCC in the U.S. (2000–2012)

1.2 Risk Factors for Hepatocellular Carcinoma

HCC is unique in that it largely occurs within an established background of cirrhosis (~70–90 % of all detected HCC cases) (Fig. 1.7). The two major causes of cirrhosis and thus HCC globally include hepatitis B (Fig. 1.8) and hepatitis C (Fig. 1.9) virus infection, which collectively occur in close to 80 % of all HCC cases [25]. Other established as well as emerging risk factors include: alcohol and tobacco use; aflatoxin exposure; obesity, diabetes, and nonfatty liver disease; and diet. The distribution and impact of HCC risk factors often varies considerably across regions, populations and time periods. The epidemiological data linking these specific risk factors to HCC in particular is overviewed below. Our overview when sufficient data exists focuses primarily on findings reported in cohort studies, particularly those that are population-based and prospective and on meta-analyses of these prospective studies as this is considered the strongest direct observational epidemiologic data in support of a potential causal association.

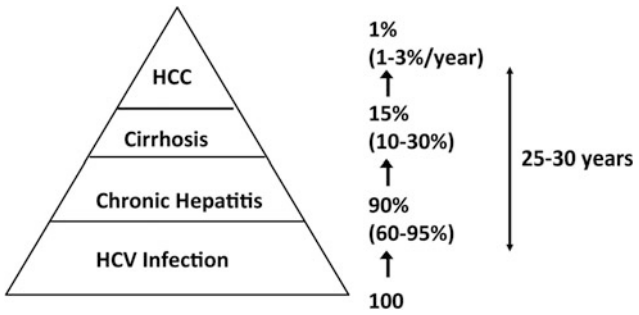

Fig. 1.7 Estimated progression rates to cirrhosis and hepatocellular carcinoma in hepatitis C infection

1.2.1 Hepatitis B Virus

Globally, HBV is the leading cause of HCC. An estimated 1 in 3 persons worldwide has been infected by HBV, and although only 5 % of these become chronic carriers, 25 % of chronic carriers develop serious liver disease like cirrhosis and HCC. Most HBV-related HCC cases occur in Asia and sub-Saharan Africa (Fig. 1.8), with China alone accounting for 73 % of the world's HBV-related HCC cases [2]. Chronic HBV infection affects an estimated 240 million persons worldwide, with more than 780,000 dying annually, primarily due to HBV-related liver disease [26].

In Asia, where HBV is endemic, infection is largely acquired by maternal–child transmission, while sibling-to-sibling transmission at young ages is more common in sub-Saharan Africa [27]. In these areas, up to 90 % of infected infants/children follow a lifelong chronic course. The pattern is different in areas with low endemicity, where HBV is typically acquired in adulthood through sexual and parenteral routes (horizontal transmission) and where with >90 % of acute infections in adults resolve spontaneously.

Epidemiological studies have demonstrated that chronic HBV carriers have a 5- to 15-fold increased risk of HCC compared to the general population. The great majority of HBV-related HCC (70–90 %) develops in a background of cirrhosis. A recent meta-analysis of 57 studies with treatment naïve HBV-infected cohorts found much higher annual HCC incidence in cirrhotics (3.16 vs. 0.10/100 person-years in cirrhotics vs. non-cirrhotics, respectively) [28]. It also found that although HCC incidence among HBV-related cirrhotics varied according to factors like gender, that it was largely similar in European and Asian populations.

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

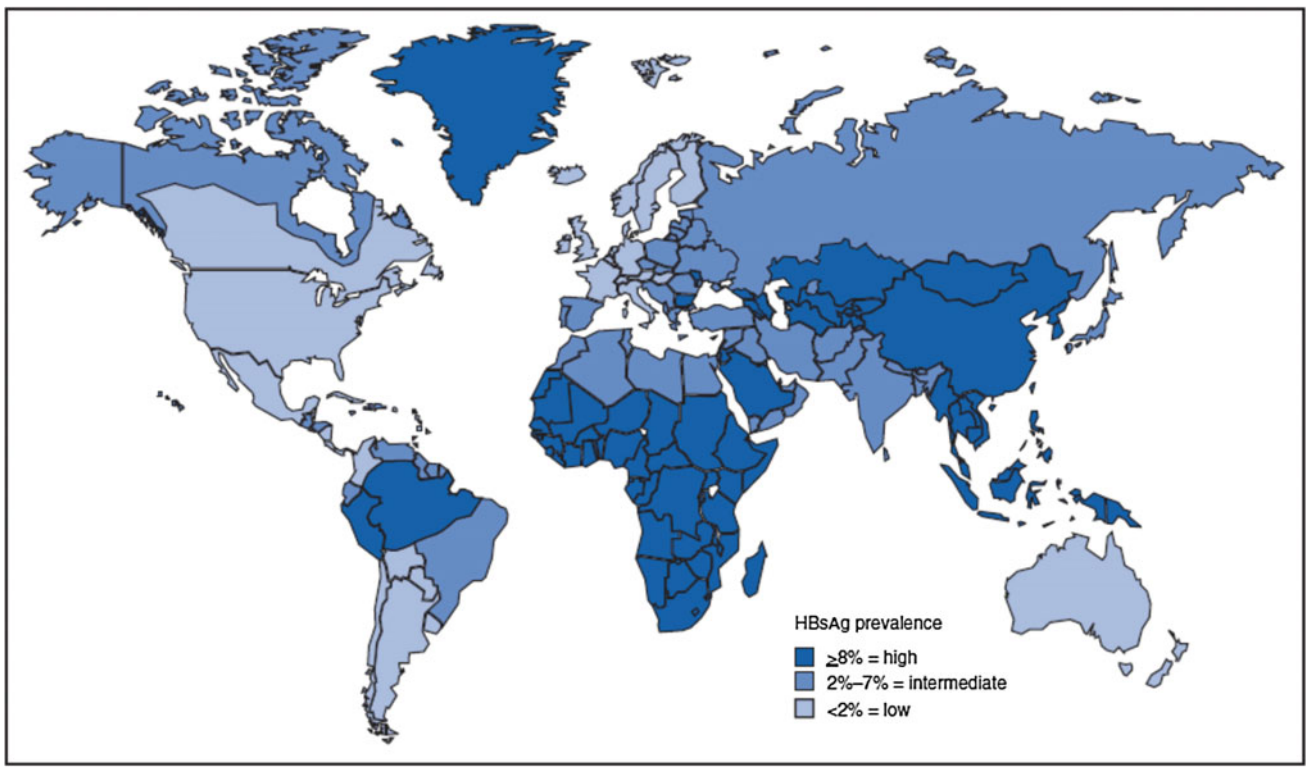

Fig. 1.8 Distribution of chronic hepatitis B virus (HBV) infection—worldwide, 2006. *Source* CDC. Travelers' health; yellow book. Atlanta, GA: U.S. Department of Health and Human Services, CDC; 2008. Available at http://wwwn.cdc.gov/travel/yellowbookch4-HepB.aspx

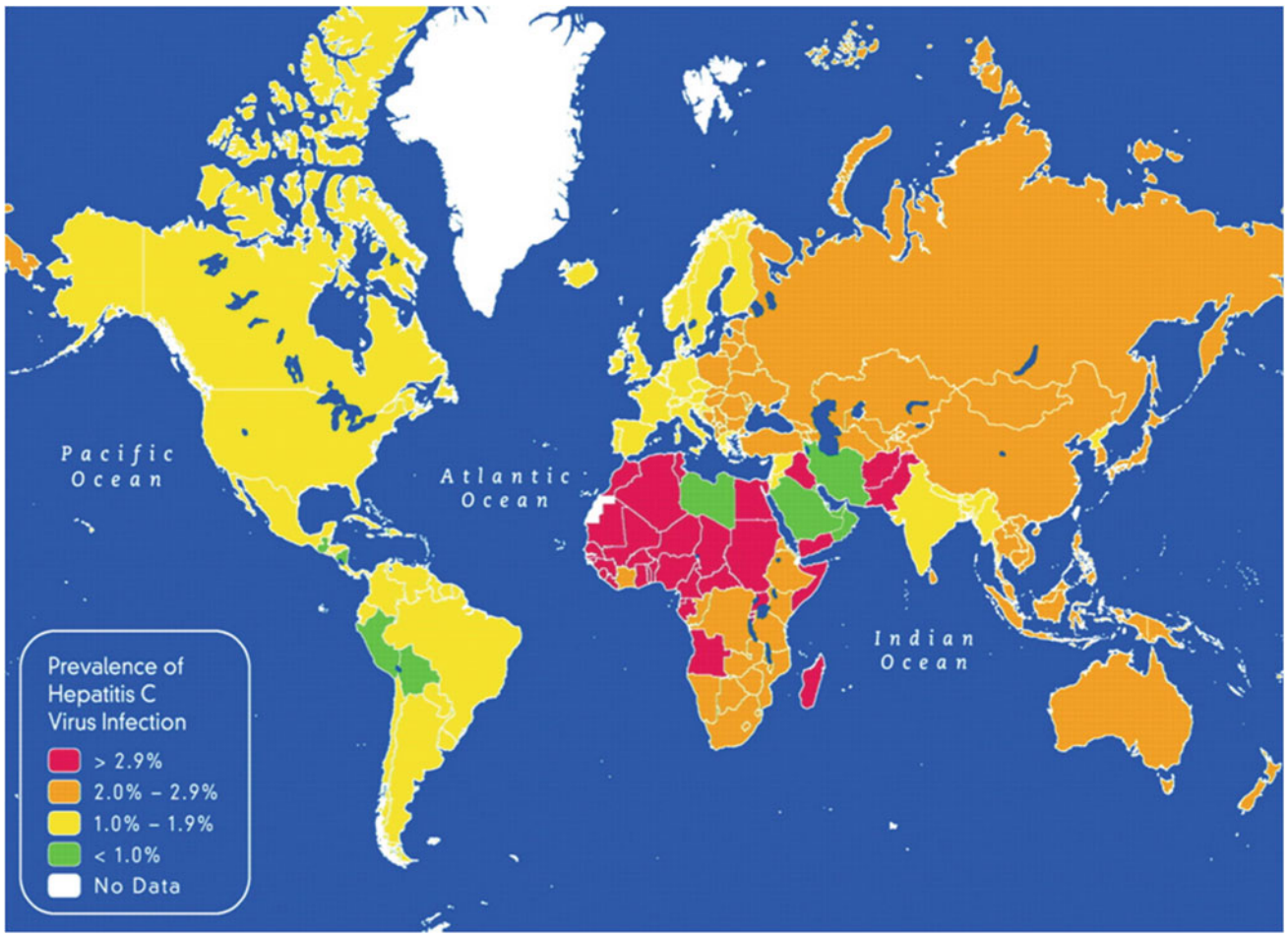

Fig. 1.9 Map of the global prevalence of chronic hepatitis C virus infection. *Source* Averhoff FM, et al. Clinical Infectious Diseases. 2012; 55: S10–S15

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 is genotype [29].

In the natural history of chronic HBV infection, spontaneous or treatment-induced development of antibodies against HBsAg and HBeAg leads to reduced HCC risk. A meta-analysis of 12 studies with 1187 patients who received interferon and 665 untreated patients followed for 5 years found lower cumulative HCC incidence in treated than untreated patients (1.9 % vs. 3.2 %, respectively), although this difference was not statistically significant [30]. However, statistically significant reduction in HCC risk was shown with use of the more recently available reverse transcriptase inhibitor medication Lamivudine, which reduced the risk of treated to untreated (odds ratio (OR) = 0.48). However, there was still notable HCC incidence during the median 43-month follow-up period among the treated (1.3/100 person-years in the treated vs. the untreated). These results are largely paralleled by results reported for other studies of single or combined use these medications and are evident in a more recent single cohort study with up to 8 years follow-up [31].

Another consideration is that that HBV DNA can persist as "occult HBV infection" for decades among persons with serological recovery (HBsAg negative). Occult HBV is associated with anti-HBc and/or anti-HBs [32]. However, in a significant proportion of individuals, neither anti-HBc nor anti-HBs can be detected. A recent meta-analysis of 8 prospective studies (6 in Asian populations) demonstrated significant increased HCC risk with occult infection compared to no infection (OR = 2.86) [33]. There was no association between occult HBV and HCC risk in the large HALT-C trial in HCV-related cirrhotics though a substantial number of cohort members had evidence of prior HBV infection.

The public health initiative in endemic countries to institute wide-scale vaccination of newborns for HBV started in the 1980s and is projected to dramatically lower HBV-associated HCC rates as that birth cohort, the eldest

now in their early 30 s, begins to age into higher risk ages for HCC onset. However, suboptimal vaccination rates and/or acquisition of immunity reported in some endemic countries or regions like Laos [34] suggests that without a viral cure, HBV-related HCC will continue to persist, albeit at dramatically lower rates, in the postvaccine generation pending discovery of a viral cure.

1.2.2 Hepatitis C Virus

HCV is the second leading cause of HCC worldwide, with most HCV-related HCC cases arising in Asia and North Africa (Fig. 1.9). Global HCV prevalence has grown over the last 15 years to over 185 million infected (~2.8 % global prevalence) [35], with recent estimated global prevalence of 46.2 % for genotype 1 (83.4 million, approximately one-third in East Asia) and 30.1 % for genotype 3 (54.3 million) [36]. The highest reported HCV prevalence in North Africa is in Egypt (~18 %) and in Asia in Mongolia (~10 %). It is also estimated that up to a million people die annually of HCV-related liver disease [37].

In contrast to HBV, vertical and early childhood infection is rare, and almost all new HCV infections arise in early adulthood. (Egypt is a notable exception; there were >5000 cases of vertical transmission in 2008 alone) [38]. Also in contrast to HBV, most infected adults (up to 80 %) develop chronic infection.

In Japan, in contrast to other high HCC rate Asian countries like China, HCV is the predominant viral cause of HCC. HCV was largely introduced there (as in Egypt) iatrogenically via use of intravenous antischistosomal therapy and began to widely disseminate shortly after World War II [39]. Consequently, HCV-related HCC rates began to sharply increase in Japan in the mid-1970s onward, although recent data suggest that the peak may already have been reached [40].

It has been estimated that HCV began to infect large numbers of young adults in North America and in South and Central Europe in the 1960s and 1970s, predominantly as a result of intravenous drug use [41]. 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. Consequently, most individuals in these chronically HCV-infected populations from developed countries have been infected for several decades and are rapidly graying into peak ages for liver disease onset. Accordingly, evidence-based model simulations suggested a peak incidence of HCV-related cirrhosis by 2020 with associated continued increases in HCC over the following decades [42]. These estimates, however, were made a few years prior to advent of highly efficacious direct acting antiviral (DAA) drug regimens that are interferon free, the first of which (sofosbuvir) became available for use the U.S. in 2014. Yet, given the very high cost of these new medications, combined with lack of awareness of underlying infection in almost all individuals with HCV infection until they are diagnosed with liver disease, their impact on the projected magnitude and timing of peak HCV-related HCC incidence rates in the U.S. (and globally) is not yet known.

HCV infection is consistently associated with substantially increased HCC risk in prospective and retrospective studies. For example, 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. However, the likelihood of development of HCC among HCV-infected persons is difficult to determine due to the paucity of adequate long-term cohort studies; the best estimate is from 1 to 4 % after 30 years (Fig. 1.9). HCV increases HCC risk by promoting fibrosis and eventually cirrhosis, with rates of cirrhosis up to 30 % [43]. after several decades of infection [44]. Once cirrhosis is established, HCC develops at an annual rate of 2–5 % [45]. However, rates up to 7 % have been reported in Japan, with the historically highest incidence among recipients of contaminated blood or blood products (14 and 1 per 1000 person-years for cirrhosis and HCC, respectively) and in hemophiliacs (5 and 0.7 per 1000 person-years, respectively), and the lowest in women who received a one-time contaminated anti-D immune globulin treatment (1 and 0 per 1000 person-years, respectively).

In HCV-infected patients, factors related to host and environment/lifestyle appear to be more important than viral factors in determining progression to cirrhosis. Some of the key factors include: older age; older age at the time of infection; longer duration of infection; male gender; heavy alcohol intake (>50 g/day); obesity, diabetes, and fatty liver disease; and coinfection with HBV or HIV (with ~16–33 % coinfected in the U.S.) [46]. Although there is no strong evidence for an effect of most viral factors like viral load or quasispecies in HCC risk, our recent cohort based research in 111,000 chronically HCV-infected veterans using VA healthcare between 2002 and 2009 demonstrated 80 % excess HCC risk among those infected with genotype 3 in comparison to genotype 1 (HR = 1.80, 95 % CI: 1.61–2.03) [47]. We also found evidence suggestive of potential racial differences in risk of HCV-related progression within this VA cohort, with significantly increased HCC risk observed in Hispanics and significantly decreased risk among African Americans (HRs = 1.28 and 0.58, respectively) [48]. Globally, the most prevalent HCV genotypes are types 1 and 3, representing 46.2 % and 30.1 % of all cases, respectively. The less common genotypes (2, 4, 5 and 6) are disproportionately found in less developed countries [35].

In the U.S. a recently reported estimate of the population attributable fraction (PAF) or proportion for of HCC cases that would be eliminated with removal of HCV as risk factor is 22.4 % among individuals aged $\geq$68 years using SEER-Medicare data for 1994–2007 [49]. The PAF is an important measure of the burden of a given risk factor within a population as it incorporates information on both strength as well as prevalence of exposure; although they may individually sum to more than 100 % with risk factor overlap. In this case although the population prevalence of chronic HCV is low (<2 %), HCV is by far the strongest HCV risk factor (ORs = 39.9, 11.2, 4.1, and 2.5 for HCV, HBV, alcohol, and diabetes/obesity, respectively, all p-values < 0.05) [49].

Prior to the DAA era, very few chronically HCV-infected patients were successfully treated, i.e., were eligible to receive and tolerate treatment and to achieve a sustained virological response (SVR). There is enhanced recent interest in the longer term outcomes in these "SVR cohorts" as they likely provide the best available estimates of the potential benefit of obtaining SVR via treatment with costly new medications. The imperfect HCC risk reduction with DAA, in conjunction with high treatment costs and lack of awareness of HCV among populations globally, has led to the call for multipronged approaches to effectively prevent, as well as treat, HCV infection and thus its associated serious sequalae like HCC.

1.2.3 Alcohol

Heavy alcohol intake, defined as ingestion of >50–70 g/day for prolonged periods, is a well-established HCC risk factor, with IARC determining there was sufficient evidence to label it a human hepatocarcinogen in 1988 [50]. Although chronic heavy intake is strongly associated with development of cirrhosis, there is little evidence of a strong direct carcinogenic effect of alcohol alone.

The association between alcohol use and HCC risk has been evaluated in numerous studies. Although higher levels of alcohol intake and alcohol abuse disorders are typically associated with increased HCC risk in most prospective cohort research particularly in general population cohorts, the magnitude of reported excess risk varies across populations and classification of alcohol use. For example, in the U.S. in the NIH-AARP cohort study of almost 495,000 general community participants, those with highest regular alcohol consumption had significant approximate twofold increased risk in multivariable analyses (HR = 1.92, 95 % CI 1.42–2.6) [51], while strong excess risk was observed in an Italian cohort of >8500 hospital discharge diagnosed alcoholics in comparison with risk based on age and gender-specific Italian population norms (standardized incidence rate ratios = 6.9 (4.5–10.0) and 5.9 (0.1–32.6) in male and female alcoholics respectively) [52].

There is also evidence suggestive of potential synergistic increases in HCC risk among those with both heavy alcohol ingestion and several other established risk factors like obesity and viral hepatitis. For example, in a cohort study in almost 24,000 Taiwanese residents (half male) from 7 townships who were followed for 11.6 years reporting an overall HR for obesity or BMI > 30 = 3.82 (95 % CI 1.94–7.52), that was substantially higher (HR = 7.19) among those with BMI > 30 and whom also were alcohol users, but that was not elevated (HR = 1.06) among obese nondrinkers [53].

Globally the prevalence of alcohol drinking varies considerably across countries, with highest per capita consumption found in more highly developed western countries especially in the Northern Hemisphere and is lowest in countries in sub-Saharan Africa and the Middle East. Approximately 11.5 % of all alcohol users worldwide have heavy drinking episodes weekly, with men outnumbering women 4:1 [54]. These large population differences in alcohol exposure are apparent in calculation of PAFs. For example, in the U.S. SEER-Medicare cohort (1973–2007), presence of an alcohol-related disorder was the 2nd leading single risk factor (PAF 23.5 %); though this varied by race/ethnic group (e.g., 25.6 % in White, 18.5 % in African Americans, 15.2 % in Asian, and 30 % in Hispanic males) and gender (e.g., 27.8 % and 15.4 % in males and females overall) [49]. However, although PAFs for alcohol-related HCC are universally lower in women globally, there is reason for ongoing vigilance and investigation in women given ongoing dramatic recent increases in drinking among women in many parts of the world in the last few decades along with some experimental and clinical data suggesting that females may be more susceptible to alcohol-associated liver damage than males.

1.2.4 Aflatoxin

Aflatoxin B1 (AFB1) is a ubiquitous mycotoxin produced by the *Aspergillus* fungus. Several staple crops, including cereal grains, tree nuts, legumes (principally peanuts), and most especially maize, are particularly susceptible to aflatoxin contamination, especially under unfavorable crop (e.g., high humidity, drought, insect infestation) and storage/processing (e.g., suboptimal harvest, drying and storage) conditions. Animal experiments have demonstrated that AFB1 is a powerful hepatocarcinogen, leading the International Agency for Research on Cancer (IARC) to classify it as carcinogenic [16]. Once ingested, AFB1 is metabolized to an active intermediate, AFB1-exo-8,9-epoxide, which can bind

to DNA and cause damage, including producing a characteristic mutation in the p53 tumor suppressor gene (p53 249ser) [55].

Over the last two decades, strong evidence that AFB1 is a risk factor for HCC in human populations has been supplied by multiple person-specific epidemiological research studies using direct biomarker assessment performed [12, 56–60]. These studies were made possible by development of assays for aflatoxin metabolites in urine and AFB1-albumin adducts in serum and by assays for detection of a signature aflatoxin DNA mutation in tissues. For example, this mutation has been observed in 30–60 % of HCC tumors in endemic areas.

Several of these studies also reported evidence of synergism between AFB1 and other known liver disease factors in promotion of HCC risk. For example, in prospective cohort research conducted in Shanghai, China, urinary excretion of aflatoxin metabolites was associated with fourfold increased HCC risk, while HBV infection risk was increased sevenfold. However, individuals who both excreted AFB1 metabolites *and* were also HBV carriers had a dramatic 60-fold syngergistic increased HCC risk [61]. AFB1 exposure has also been found to synergistically increase HCC risk in conjunction with other known risk factors like HCV, obesity, alcohol and smoking, although these synergistic effects were not as strong as those reported with HBV. However, a more than additive, rather than a multiplicative, synergism with HBV has recently also been suggested.

Although most substantial AFB1 exposure is presumptively related to dietary intake, a recent case-control study in China found over half of sugar warehouse and paper production factory workers (1993–2004) had detectable urinary AFB1 DNA adducts compared to only 12 % of non-factory worker controls [56]. In addition, workers with highest adduct levels had over a fivefold significant HCC risk compared to those with lower levels (OR = 5.24; 95 % CI: 2.77–9.88; $P = 0.00$).

Globally, it is estimated that up to a quarter of all agricultural products are aflatoxin contaminated. However, the likelihood of dietary aflatoxin intake above maximum guideline-recommended levels is predominantly concentrated in southeastern Asia and sub-Saharan Africa, largely overlapping areas where HBV is also endemic. Efforts to reduce aflatoxin exposure via a variety of methods like switching from maize to less AFB1 susceptible crops, public health education, and government regulation began in the early 1980s in multiple high regions including in China, Taiwan and Africa [13].

In both China and Taiwan, these efforts have already reaped notable dividends. Reduced HCC risk has been observed, particularly among birth cohorts antedating government-mandated neonatal HBV vaccination: a 1.9-fold risk reduction among 25–29 years olds in 1990–1993 compared to 1980–1983 and a 1.4-fold reduced risk among 40–44 year olds in 2005–2008 compared to 1980–1983 in Qidong City, China and an estimated overall 65 % reduction in primary liver cancer due to government initiated switch from maize to rice [12, 62]. There has also been substantial reduction in PAF for HCC conveyed by AFB1 in HBV + Taiwanese populations from 31 % in 1980s to 12 % by 1990s [63]. However, in many areas, including in Africa and in more rural areas of China, AFB1 exposure is still problematic; e.g., 78 % prevalence of serum AFB1-lysine among 500 randomly selected individuals from a nationally representative cross-sectional survey across Kenya in 2007 [58]. A recent systematic and meta-analysis suggested that in these areas the PAF for HCC from AFB1 exposure is 17 % overall (14–19 %), and is synergistically higher (21 %) in populations with HBV compared to those without (21 % HBV + -AFB1 + vs. AFB1 + HBV- populations, respectively) [64]. The lack of success in reduction of AFB1 exposure in some high-risk regions (particularly in Africa) [65, 66] has been attributed to a variable range of factors, including lack of regulation and/or enforcement, cultural practices, lack of knowledge about risks, inadequate public health infrastructure, and costs [67]. However, the ongoing high burden of HCC in these areas, combined with other known deleterious impacts of AFB1 intake, including growth retardation, suggests the need for enhanced efforts to reduce exposure risk.

In contrast to Asia and Africa, AFB1 is not considered to be a risk factor in developed nations in Western Europe and North America which have strict enforcement and regulation of food production and acceptable AFB1 thresholds which are applied to food both domestically produced and imported foods [68]. It is estimated that current standards reduce risk of a dietary consumption from primary AFB1 source, peanuts, are sufficient to limit AFB1-associated HCC occurrence to 1 in 10,000 persons [69].

However, a recent study in Bexar County, Texas, which is close to the Mexican border and which has among the highest HCC rates reported in the U.S., found 21 % of healthy participants had detectable AFB_1-albumin adducts. In earlier research, 23 HCC patients, predominantly non-Hispanic Caucasians from Texas and Louisiana, were seen (1986–1994) at the MD Anderson Cancer Center (Houston, Texas). In half without evidence of viral hepatitis, detectable levels of AFB_1-DNA adducts were found in three of 19 tumors tested, with 2 of 3 in males without evidence of viral infection. These data, combined with largely unexamined role of AFB1 as a potential primary or secondary contributor to HCC in the U.S., suggests the need for additional surveillance and research in the U.S., particularly among subgroups that may potentially have or had elevated AFB1 exposure (e.g., rural residence, agricultural production, recent immigration from AFB1 high-risk regions).

1.2.5 Diabetes

Diabetes, particularly type II, has been proposed to be a risk factor for HCC, particularly via its close association with NAFLD and cirrhosis [70]. Specifically, diabetes is known to contribute to hepatic steatosis, with increasing levels of steatosis associated with more severe necroinflammatory activity and fibrosis. There is also experimental evidence that diabetes can promote HCC risk more directly, for example by favoring formation of hepatic DNA damage from reactive oxygen species generated by diabetes-associated increases in levels of inflammatory cytokines like IL-6 and TNF-α [71] and from advanced glycation end products [72]

Across diverse epidemiologic designs (case-control, cross-sectional, and cohort), settings (clinical and community) and populations, almost all studies report that type 2 diabetes is associated with excess risk, most in the moderate range (1.5–3.5-fold excess). A recent meta-analysis, synthesizing results from 21 prospective cohorts studies reported through fall 2013, calculated a pooled 86 % increase in relative HCC risk among diabetics, an effect consistent with the reported pooled estimate from retrospective case-control and cross-sectional studies (1.86; 95 % CI:1.49–2.31) [73].

Although the consistency and strength of these prospective cohort findings are supportive of diabetes being a potential etiopathogenic risk factor, the findings of these studies may be influenced by a potential reverse causation bias, given the liver's role in glucose metabolism. Specifically, liver disease itself negatively impacts host glucose metabolism, favoring increased insulin resistance; therefore diabetes may be both a result of, as well as a cause of, advanced liver disease. This problem is particularly relevant in evaluating diabetes as an HCC risk factor because 10–20 % of patients with cirrhosis have clinically diagnosed diabetes, with a much larger percentage having impaired glucose tolerance.

Further complicating this picture, in the case of HCV infection, our earlier meta-analysis of prospective cohort studies suggests that HCV infection itself also independently increases risk of diabetes [74], a finding also consistent with much experimental research. However, moderate excess diabetes-associated risk persists among most prospective cohort studies that adjusted for baseline level of liver disease determined by biopsy or serological marker.

Additional data suggestive of a potential etiopathogenic role for diabetes comes from multiple pharmacoepidemiology case-control and cohort studies that found use of the antidiabetic medication metformin was associated with moderate to strong significantly decreased (decreased) HCC risk [75–79]. These results appear to raise the intriguing possibility that metformin, a widely used antidiabetic with a generally benign side effect profile, may have potential benefit as a chemopreventive agent in subgroups at high HCC risk. However, there are several reasons for cautious interpretation of the pharmacoepidemiological data currently available. In addition to the limited total number of studies (particularly of large directly measured prospective cohorts), most had methodological limitations, like failure to account for differences in individual patient propensity to receive metformin, not accounting for medication adherence (e.g., via calculation of a medication possession ratio) and inadequately accounting for time window, time lag, and/or dose duration of medication use that could systematically bias results. Nonetheless, the consistency of these findings with several experimental reports of anti-HCC tumor effects of metformin [80] supports the need for additional research on its potential value as a HCC chemopreventive.

A few pharmacoepidemiological studies have also evaluated use of hyperglycemics insulin and sufonlyureas; a recent meta-analysis including both prospective and case-control studies reported 1.6-fold excess HCC risk with sufonlyurea use (n = 8 studies) and 2.6-fold increased risk with insulin use (n = 7 studies) [81]. However, as these medications are indicated for use in individuals with more advanced diabetes, unmanaged by other means including diet or metformin, it is unclear whether this excess risk is due only to a potential reverse causation bias or whether these medications may also convey additional independent HCC risk.

Overall, it is estimated, based on SEER-Medicare data 1973–2007, that the elimination of diabetes and related metabolic syndrome features like obesity in countries like the U.S., which has among the highest global obesity rates but low rates of viral infection, would have a greater impact on HCC reduction than that of other strong, though less prevalent, risk factors like HCV (e.g., estimated PAF = 36.6 % diabetes/obesity vs. PAF = 22.4 % HCV in U.S) [49].

1.2.6 Obesity

Obesity, particularly abdominal or visceral, is closely correlated with increased insulin resistance and Type 2 diabetes risk. Like diabetes, it has been posited to increase HCC risk predominantly by promoting development and progression of hepatic fibrosis. However, experimental research suggests it may also increase HCC risk by other diverse mechanisms, for example, by increasing epigenetic aging in hepatocytes [82] and by promoting gut microbial changes that favor formation of hepatotoxins [83].

Numerous case-control and cross-sectional studies have evaluated the association between phenotypic adiposity, most typically assessed using BMI-based measures, and HCC risk

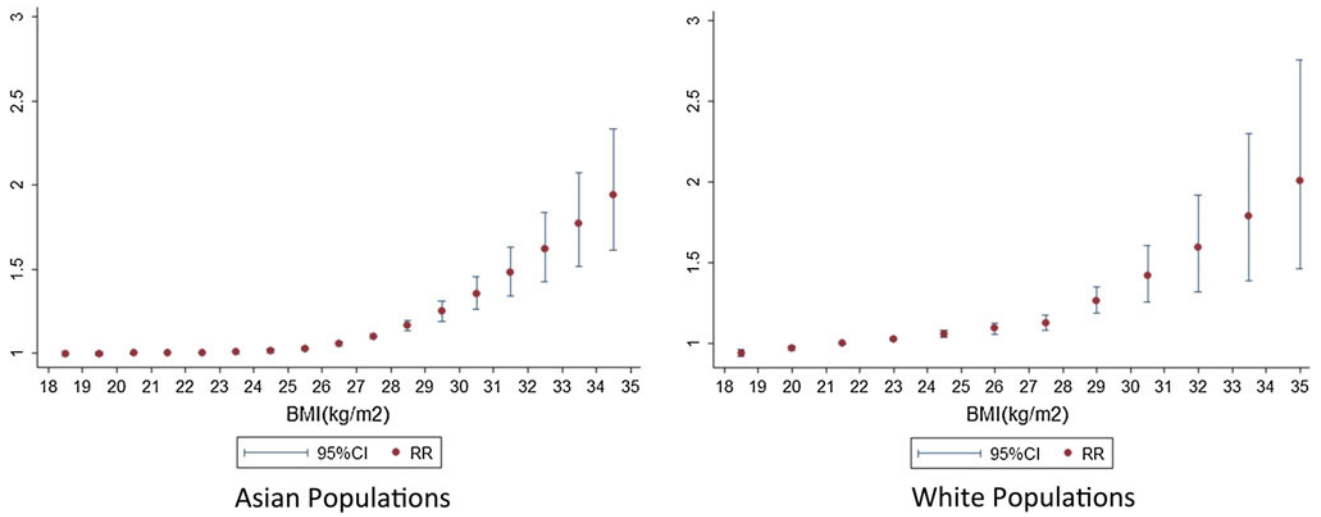

Fig. 1.10 Association between BMI and relative risk of HCC reported in prospective cohort studies in Asian and White Populations (adapted from Rui et. al 2012)

(Fig. 1.10). Obesity when defined as BMI > 30 has almost uniformly been associated with elevated incidence rates in large population cohort studies, whether conducted in low, medium or high-risk cohorts. A recent meta-analysis of 11 prospective cohort studies reported a significant overall 1.89-fold excess risk (95 % CI: 1.51–2.36) with obesity and lower though significant pooled 1.17-fold increased risk (95 % CI: 1.02–1.34) with overweight [84]. There is increasing epidemiological evidence that the presence of obesity with another established risk like alcohol, HBV or HCV infection, AFB1, and/or smoking results in particularly elevated HCC risk. However, the underlying explanatory mechanisms for this interaction or synergism are not known.

There is less data on the association between other anthropometric measurements of obesity. A single nested case-control study conducted in the European EPIC cohort study found that the waist-to-height ratio (WHtR) was the strongest anthropometric HCC risk factor; in multivariable analysis, individuals with WHtR in the highest versus the lowest tertile had a relative risk (RR) = 3.5 (95 % CI: 2.09–5.87; p-trend < 0.00010) [85]. Weight gain in adulthood was also associated with significant 2.5-fold excess risk when comparing the highest to lowest tertiles in the EPIC cohort [85].

Weight loss has been found in biopsy-based studies of obese patients with nonalcoholic steatohepatitis to improve histopathology, with significant improvements seen even with modest weight loss and dramatic reversals in many bariatric surgery patients. Yet there is limited data on the association between substantial weight loss among the obese and subsequent HCC risk, with a single-center study of 14 obese Australian patients with incidental cirrhosis diagnosed at time of laparoscopic gastric banding reporting that two incident cases of NAFLD-related HCC arose during the median 64.5 month follow-up period [86]. However, lack of a comparison group coupled with the small sample size, limit inferences on the potential impact of obesity on HCC risk within even this single study population.

There is also limited data on the potential impact of childhood obesity. Although HCC occurs almost exclusively in late middle-aged and older adults, case reports of presumptive obesity-associated HCC have been reported in young adults and recently in a 7-year-old obese child [87]. A single population-based cohort study of >285,000 Danish school children aged 7–13 years old, born 1930–1980 and followed through 2008, assessed the association between childhood BMI and HCC risk. It found similar significant excess HCC risk among both boys and girls with increasing BMI, whether using BMI whether measured at ages 7 or 13 [88]. However, the applicability of these findings to other populations, particularly those with different racial/ethnic composition and with much higher rates of childhood obesity is unknown. Given the escalating global childhood obesity pandemic, however, its potential association with adult HCC risk bears further systematic investigation. Finally, although the full impact of increasing childhood obesity on HCC rates within specific populations will likely not be fully understood for several decades, it may well be evident first in Western populations where childhood obesity rates rose first, fastest, and most substantially.

Overall, the PAF or proportionate reduction in HCC cases among adults $\geq$68 years with removal of obesity + diabetes in the U.S., estimated using SEER-Medicare cancer registry data, is 36.6 % [49]. This is not a surprising finding,

given high age-adjusted population prevalence rates of obesity in U.S., ~68.5 % with BMI ≥ 25 reported by the CDC in 2015. However, even in high HCC rate countries like China and South Korea where the proportion of adults with BMI ≥ 25 is much lower than in the U.S. (34.4 % in China and 33.5 % in South Korea based on 2010 WHO estimates), the impact of obesity reduction on HCC burden is still likely substantial, because obesity similarly increases HCC risk in Asian as in White populations based on meta-analysis of prospective cohort studies (Fig. 1.10), and because of the observed synergism between obesity and HBV which is more prevalent in many Asian populations. Finally, even though the absolute lifetime risk of HCC associated with obesity particularly in the absence of other known risk factors is low, and the potential causal mechanisms are less clear, given global obesity rates continue rapid increase, the proportion of HCC cancers that are obesity and metabolic syndrome-associated is projected to continue to rise worldwide over the coming decades.

1.2.7 Nonalcoholic Fatty Liver Disease (NAFLD)

A substantial and growing minority of HCC cases reported in the epidemiological literature has been attributed to NAFLD or to cryptogenic disease, which is often presumptively NAFLD-related given the high associated prevalence of metabolic syndrome features. NAFLD is characterized by excess triglyceride accumulation in liver cells (steatosis) in the absence of excessive alcohol intake. It is posited as the hepatic manifestation of the metabolic syndrome, and as such is closely associated with diabetes, obesity, dyslipidemia and hypertension. Although typically a nonprogressive condition, at least 20–30 % of NAFLD patients progress to nonalcoholic steatohepatitis (NASH) which is characterized by liver cell injury, inflammation and fibrosis, and can result in cirrhosis in 10–20 % of cases [89].

We previously performed a systematic review to assess the association between NAFLD, NASH, and cryptogenic cirrhosis presumed to be NASH-related, and the risk of HCC. We analyzed data from a total of 17 cohort studies (6 U.S., 6 Europe/Australia/multi-Western countries, 5 Japan) and found that NAFLD or NASH cohorts with few or no cases of cirrhosis cases at baseline had a minimal risk for HCC (cumulative HCC mortality of 0–3 % for study periods up to 20 years, most reporting 0–1 %) [23]. In contrast, cohorts with NASH and cirrhosis at baseline had a consistently higher reported risk (cumulative incidence ranging from 2.4 % over 7 years to 12.8 % over 3 years). However, reported risk estimates for HCC in fatty liver disease-associated cirrhosis cohorts were considerably lower than those reported in similar HCV-related cirrhosis cohorts. We also found in our parallel review of 18 case-control and cross-sectional studies (8 in Asia, 6 in Europe, and 4 in the U.S.) that prevalence of cirrhosis among HCC cases attributed to NAFLD/NASH was variable, ranging between 36 and 90 %, with three-quarters reporting rates ≥ 70 % [23].

More recently, a large U.S. population-based cohort study utilizing the NCI's SEER-Medicare registry reported that 14.1 % of HCC cases were NAFLD-related, with an average annual 9 % increase in rate of NAFLD-related HCC between 2004 and 2009 [90]. These findings are consistent with reported doubling of NAFLD prevalence in the general population over the last two decades to ~30 % [91], with NAFLD now the leading cause of chronic liver disease [92] and the fastest rising cause of cirrhosis in the U.S. [93, 94].

Historically, NAFLD has been considered a disease that predominantly afflicted developed Western countries like the U.S. However, notable and also increasing prevalence of NAFLD has also been reported in diverse populations across North, South and Southeast Asia, with typical recent prevalence estimates between 15 and 20 % [95, 96]. In spite of several crucial developments that have or are projected to substantially reduce the global burden from viral hepatitis, like national neonatal HBV vaccination and the advent of highly efficacious DAAs for HCV, the growing global obesity pandemic suggests that NAFLD-related HCC will increase and also represent an increasingly large proportion of HCC cases in many populations worldwide in coming decades.

1.2.8 Tobacco

The IARC found sufficient evidence to classify tobacco smoking as a cause of human HCC in 2004, while the U.S. Surgeon General classified smoking as a probable cause in 2014. Results from a recently reported meta-analysis of 27 cohort studies (many performed since 2000) offer further support for that designation, with significant pooled HRs for HCC risk of 1.45 (95 % CI 1.33–1.59) and 1.22 (95 % CI 1.11–1.34) for current and former smokers, respectively, compared to nonsmokers. A dose–response effect was also evident with a significant 7.1 % increase in HCC risk for each ten additional cigarettes smoked daily. Although only a minority of these studies specifically reported gender-stratified results, all demonstrated that smoking-associated excess risk was greater in women than in men. However, the extent to which this difference reflects true underlying biological sex-based differences in susceptibility to tobacco-induced liver injury versus reflects the combined effects of much lower background risk for HCC and much lower prevalence of smoking among women is not well-understood.

There is also evidence suggesting that smoking may be associated with markedly elevated excess HCC risk in

conjunction with some other established risk factors. For example, a large study (35,784 participants from four population-based cohort studies, subjects recruited 1977–1993 and followed through 1998) found cumulative HRs for HCC risk of 7.53 (95 % CI: 2.93–19.35) for obese heavy smokers, 3.90 (95 % CI: 1.55–9.80) for obese light smokers, and 2.21 (95 % CI: 1.18–4.15) for obese nonsmokers compared to non-obese nonsmokers, respectively [97]. Additionally, more than additive synergism between smoking and HBV and more than multiplicative synergism between smoking and HCV on HCC risk was demonstrated in a recent meta-analysis of prospective cohort studies [98]. The exact mechanisms underlying these large joint effects, including why the synergism with smoking may be stronger with some risk factors compared to others, are not established.

According to World Health Organization (WHO) data, 36 % of males and 7 % of females aged 15 and over smoked tobacco in 2012. The global burden of smoking, however, is not evenly distributed, with 80 % of all smokers living in low and middle-income countries and with many of these countries having smoking rates that have substantially increased over the last two decades. Currently, the highest estimated PAF for HCC conveyed by smoking is in Europe (47.6% based upon the EPIC cohort study) [99]. This is not surprising given the moderate to high rate of smoking among males in many European countries (e.g., Germany 34 %, France 31 %, Greece 53 %), that Europe has the highest reported prevalence of smoking among women worldwide (19 % overall, with smoking rates in women in many European countries only modestly lower than those in males), and the generally low prevalence of chronic HCV and HBV infection.

However, as many countries that have the highest rates of viral hepatitis infection also have high and increasing rates of smoking, smoking represents a substantial preventable cause of smoking among males. In China, for example, where the WHO estimated prevalence of smoking in males is ~44 %, the estimated attributable fraction or proportion of HCC cases that would be avoided if smoking was removed is 18.7 % [100]. For women in China, the estimated 1 % PAF for smoking predominantly reflects the low rate of smoking in Chinese women overall (~1.9 %). However, this may well be an underestimate of the smoking-associated HCC burden in women, given they may well be exposed to substantial amounts of second-hand smoke, with the high prevalence of smoking among males, and given that smoking rates in younger Chinese women have also risen substantially in recent years.

1.2.9 Oral Contraceptives

Given the pronounced gender difference in HCC incidence between men and women, the role of exogenous estrogen exposure from oral contraceptives (OC) has been raised. Use of OCs is a well-recognized risk factor for hepatocellular adenomas (HCAs), benign liver tumors. Although the IARC in 1999 classified the (early) high dose OC as a causative factor for liver cancer, based on experimental animal data and results from the few case reports and results of some (though not all) of the few epidemiology studies of women from the 1970s through the early 1980s (and probably out of an abundance of caution), that conclusion has been, and remains, the subject of much debate. In the interim, OC formulations have changed substantially and studies have been contradictory; in fact, a more recent meta-analysis of some of the same data used initially by IARC reported the data to be inconclusive [101]. Recently, the largest prospective study, which included almost 800,000 U.S. women in 11 cohorts, reported neither strong nor significant excess risk of HCC with OC use (HR = 1.12, 95 % CI = 0.82–1.55) [90, 102]. The only hormone-related factor significantly associated with HCC in this study was early bilateral oophorectomy (ovary removal), which resulted in significant 2.6-fold excess risk. The same finding, including the same significant 2.6-fold excess risk, was also found in an earlier study of HCC + Taiwanese women [103]. That study reported a statistically nonsignificant decreased risk of HCC with use of OCs. Interestingly, however, it also reported a significant decreased risk (multivariate-adjusted OR 0.46; 95 % CI, 0.27–0.79) from use of postmenopausal hormone replacement therapy (HRT). It therefore appears that further study of the type and timing of exogenous estrogenic compounds could be helpful in understanding the role of female hormones in HCC risk.

1.2.10 Coffee, Tea, and Other Dietary Intake

Coffee: Coffee is the most frequently studied customary dietary factor, with most individual studies reporting modest to moderate though variably significant decreased risk. A 2013 meta-analysis of 16 studies demonstrated that coffee drinkers overall had a significant moderate 0.6-fold reduction in relative HCC risk compared to non-coffee drinkers based on eight pooled cohort study findings, and a highly similar 0.56-fold reduction based on eight pooled case-control findings [104] (Fig. 1.11). More recent reports from several large population-based prospective cohort

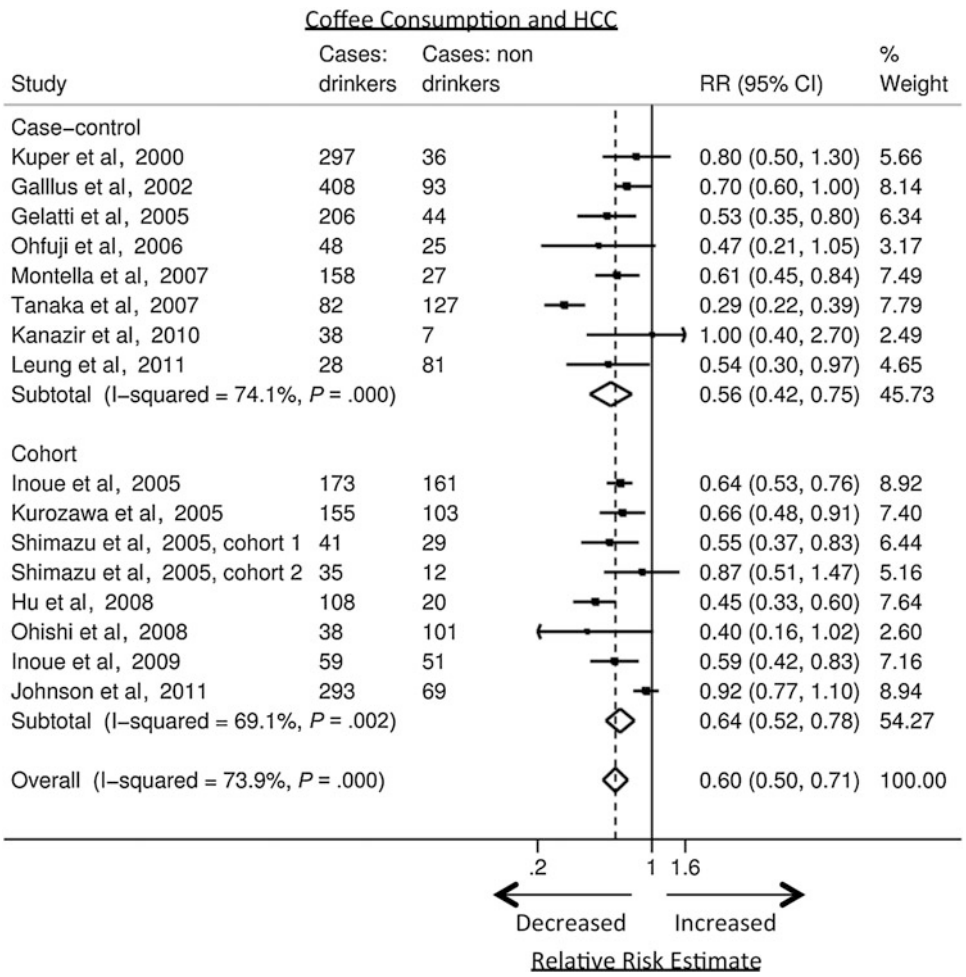

Fig. 1.11 Meta-analysis results for study-specific and summary RRs for HCC risk in coffee drinkers versus nondrinkers (adapted from reported results in Bravi et al. 2013)

studies further suggest that moderate to strong reductions in relative HCC risk is primarily found in individuals with high levels of daily coffee consumption. In the Singapore Chinese Health Study, cohort members drinking 3 or more cups of coffee daily had significant 0.56-fold reduced risk compared to non-coffee drinkers after adjusting for other liver disease risk factors [105], while in the European EPIC cohort, members with daily coffee consumption in the highest quintile had significant 0.28-fold risk reduction compared to those in the lowest quintile) [106].

In contrast, decaffeinated coffee has been consistently found to not be associated with decreased risk in most research conducted in large and prospectively followed cohorts [106, 107]. Whether this indicates that the reduced HCC risk observed with regular coffee is predominantly caffeine-related, or whether it is partially (or even predominantly) due to one of the hundreds of other known chemical constituents of coffee, like chlorogenic acid (a known antioxidant and a major phenol in coffee) or diterpenes (like cafestol) that might also be adversely impacted by the decaffeination process, is unclear.

A recent biomarker-based coffee-HCC mediation analysis performed within a nested case-control study of 125 HCC cases and 250 matched controls from the EPIC cohort suggests that the observed coffee-associated reduction in HCC risk with high daily intake is predominantly explained by the significant relationship between the amount of coffee consumed and circulating levels of six biomarkers, including inflammatory/immune response related IL-6 and hepatocellular/cholestatic injury related GLDH, ALT, AST, GGT, and bilirubin [108]. All were independently associated with HCC risk (e.g., each cup of daily coffee consumed was associated with $\beta = -0.18$ decrease in IL-6 levels for each additional cup of coffee consumed, $p = 0.04$).

The strength and consistency of epidemiological findings for coffee have led some to consider whether coffee is ready to be actively prescribed by physicians as a functional food or chemopreventive for HCC. However, others have called for a more cautious and qualified approach [109], including additional research to address important unanswered questions and concerns including: (1) what is an optimally effective and yet minimum dose of coffee necessary for

reduced risk and how do differences in coffee preparation (e.g., espresso, filtered, or instant; roasting; type of coffee bean) influence "dosing"; (2) what is the optimal timing and minimum duration of coffee drinking necessary to convey benefit, including whether coffee consumption that begins only after liver disease is well-established still conveys substantial benefits, and (3) potential barriers to prescribed use of coffee in some patients and populations based on clinical concerns, tolerability, economic feasibility in some populations, and other cultural dietary considerations.

Tea: Meta-analyses that have compared tea drinkers to nondrinkers have reported no association with risk. However, these analyses combined all types of tea (e.g., green, black, herbal) [110, 111]. The most extensively studied is green tea intake in Asian populations. A meta-analysis pooling results of 11 prospective Asian cohort studies reported that highest daily green tea intake was associated with modestly decreased HCC risk, although the risk reduction was stronger as well as significant only in women (OR = 0.78, $p < 0.05$ and OR = 0.89, $p = 0.05$ for highest vs. low/no daily green tea intake in women and men, respectively) [112].

However, concern has arisen that green tea intake in chronic HBV patients may not be beneficial, and may in fact be harmful, based on findings from a recent biomarker-based nested case-control study performed within the large Shanghai Cohort Study population. Specifically, it found that individuals who were both HBV surface antigen positive and had higher baseline urinary levels of epigallocatechin (ECG) had significantly increased risk of developing HCC (p-value for trend with increasing consumption < 0.01), and that this ECG-associated excess risk was significant and particularly strong among individuals who also had lower serum retinol levels at baseline (OR = 2.62; 95 % CI 1.25–5.51) for chronic HBV individuals with detectable ECG and low retinol versus chronic HBV but non-detectable urinary ECG and normal retinol levels) [113]. Given green tea is the major dietary source of ECGs, additional biomarker-based research on the association between green tea intake and HCC risk, particularly in HBV-infected cohorts in Asia where levels of green tea consumption are highest, appears to be warranted.

Other Dietary Intake—Vegetables and fruits are among the most extensively studied other dietary factors in association with HCC risk. A 2014 meta-analysis of 19 studies that included a total of 1.29 million participants and in which 3912 incident HCC cases arose found that with each 100 g increase in daily vegetable intake there was significantly decreased risk of developing HCC (e. g., [113] OR = 0.92, 95 % CI: 0.88–0.95 among cohort studies) [114]. In contrast, increased fruit intake was not associated with HCC risk in pooled analyses of either cohort or case-control studies [114]. Red meat intake has also been examined in several case-control and cohort studies, with variable and typically nonsignificant associations reported, and with a recent meta-analysis pooling results from nine studies finding higher red meat intake was not associated with either strong or significant excess HCC risk (OR = 1.10, 95 % CI 0.85–1.42 for individuals with highest daily red meat intake compared to the lowest) [115]. In contrast, both higher daily white meat and fish intake were associated with significantly decreased HCC risk in recent meta-analyses (OR = 0.69, 95 % CI: 0.58–0.81 based upon 10 studies and OR = 0.78, 95 % CI: 0.67–0.90 based upon 7 studies for highest vs. lowest daily white meat and chicken intake, respectively) [115]. Almost all individual studies also reported decreased risk. Daily dietary fat intake (although variably classified) has also been examined in several recent large population-based prospective cohort studies. Some key findings include: a significant 1.9-fold excess HCC risk in individuals with higher baseline daily saturated fat intake and 1.5-fold excess risk with higher omega-6 polyunsaturated fatty acid (PUFA) intake reported in the U.S. NIH-AARP cohort study (n = 495,006) [116]; that increased total fat intake was associated with significantly reduced risk (per 10 g/day, HR = 0.80, 95 % CI: 0.65–0.99)—though this effect was predominantly accounted for by high levels of monounsaturated fat intake reported in the European EPIC cohort study, [117] and significant ~0.6-fold reduced HCC risk in individuals with highest intake of n-3 PUFAs (EPA, DPA and DHA) reported in a population-based cohort of 90,296 Japanese adults [118]. Dietary soy intake has been evaluated in a few large prospective studies, all conducted in Asian populations, with mixed reported results. For example, higher dietary intake of both miso soup and tofu were associated with significant 0.5-fold reduction in relative HCC risk in univariable analyses, although neither association remained significant in multivariable analysis in a nested decedent case-control study of 176 biopsy confirmed HCC cases diagnosed 1964–1988 and 560 controls dying from non-liver causes from among a cohort of >120,000 residents of Hiroshima and Nagasaki in 1945 (the REFR LSS cohort) [119]; while highest miso soup intake ($\geq$2 bowls/day) was not associated with risk in the large Japan Collaborative Cohort Study for Evaluation of Cancer (JACC) study (HRs = 1.08 and 0.95 for men and women, respectively) [120]. Of particular concern, therefore, are results from a population-based Japan Public Health Center-based Prospective Study Cohort (JPHC) of almost 20,000 adults aged 40–69 recruited between 1990 and 1993, with dietary data collected at baseline, and with median 11.8 year follow-up, that reported that in women that soy isoflavones genistein and daidzein dose dependently increased risk HCC risk after adjusting for other risk factors for liver disease including viral hepatitis, with HRs for highest versus lowest levels of 3.19 (95 %CI = 1.13–9.00, p

(trend) = 0.03) and 3.90 (95 % CI = 1.30–11.69, p (trend) = 0.01), respectively [121]. In contrast to women, soy isoflavones were not associated with HCC risk. Additional prospective research studies with adequate sample size to similarly perform multivariable analyses accounting for other HCC risk factors like viral hepatitis are needed to replicate and further investigate the potential association of soy and HCC. Finally, recent and prospective data on the association between use of supplemental vitamins and HCC risk is sparse. However, use of supplemental vitamins E and C as well as of multivitamins was examined in the combined 132,837 adults from the Shanghai Women's Health Study Cohort (recruited 1997–2000) and Shanghai Men's Health Study Cohort (recruited 2002–2006), the largest study performed to date [122]. Results for vitamin E, whether from supplemental vitamin E use or from dietary sources, consistently indicated higher levels significant reduced risk (e.g., HR = 0.52, 95 % CI 0.30–0.90 for supplemental vitamin E) with reduced risk evident both in those with and without a self-reported history of liver disease or a family history of liver cancer. In contrast, use of supplemental vitamin C or of multivitamins was associated with significantly increased risk in those with a self-reported liver disease or family history of liver cancer, whereas increased vitamin C intake from dietary sources was not associated with liver cancer risk. These findings suggest the importance of additional research on use supplemental vitamin E and C and multivitamins and HCC risk in other populations, both to replicate the findings in other high-risk Asian populations and to assess is similar are found in other populations where risk factors like obesity and diabetes are more prevalent and HBV prevalence is low.

1.3 Summary

The epidemiology of HCC suggests it is an unusual disease as it is favored under conditions of economic deprivation (e.g., AFB1, HBV, HCV) and affluence and economic development (e.g., obesity, smoking). Because the strongest HCC risk factors are viral, it is currently most similar to cancers with a single primary infectious cause (e.g., stomach cancer and *H. pylori*) in that the global burden is overwhelmingly concentrated in less developed countries and among disadvantaged subgroups within more affluent populations. Yet particularly as it relates to risk from behavioral causes like obesity, HCC is more like colorectal cancer in that it historically first and disproportionately occurred in more developed westernized societies. In more affluent societies, HCC also is a disease of disparity as it disproportionately impacts disadvantaged populations, who typically have highest levels of both viral and nonviral risk factors.

This is an exciting era particularly as it relates to the potential prevention of new HBV infections worldwide and successful viral cure for HCV and adequand adequate suppression of HBV. However, given the daunting economic and logistical barriers to successful universal use coupled with the absence of either a vaccine for HCV prevention or curative medications for HBV, both are likely to continue contributing substantially to global HCC burden for years to come. However, even if HBV and HCV were successfully eradicated worldwide, a substantial number of HCC cases would still arise, both in countries where viral hepatitis prevalence is low like the U.S. where HCC rates have tripled over the last 30 years and an estimated ~30 % of cases are not virally related and in countries where viral hepatitis rates were highest given greater risk of exposure to known environmental risk factors (e.g., AFB1 in sub-Saharan Africa) coupled with rapid large recent increases in rates of behavioral risk factors (e.g., smoking, obesity in Asia). Together this suggests the growing global obesity pandemic may ultimately supplant viral hepatitis as the most important driver of HCC trends worldwide in coming decades, with HCC continuing to be a disease impacted by both relative levels of societal affluence and deprivation, yet disproportionately impacting more disadvantaged populations and subgroups worldwide.

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Environmental Carcinogens and Risk for Human Liver Cancer

2

John D. Groopman

Contents

2.1 Introduction

Collectively, liver cancer, including hepatocellular carcinoma (HCC) and cholangiocarcinoma, accounts for 9.1 % of all reported cancer deaths and is the second most common cause of cancer mortality worldwide [1]. The incidence of liver cancer varies enormously globally and unfortunately the burden of this nearly always fatal disease is much greater in the less economically developed countries of Asia and sub-Saharan Africa (Fig. 2.1) [2]. HCC is also the most rapidly rising solid tumor in the US and Central America and is overrepresented in minority communities, including African-Americans, Hispanic/Latino-Americans, and Asian-Americans [1, 3, 4]. Overall, there are more than 750,000 new cases each year and more than 300,000 deaths annually in the People's Republic of China (PRC.) alone [2]. 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 40–49 years of age in men and between 50–59 years of age in women [5–7]. 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 well described and worldwide the number of cases among men were 554,000 and 228,000 among women in 2012 [8]. These epidemiologic findings are also reflected in experimental animal data for one potent liver carcinogen linked to human HCC, aflatoxin, where male rats have been found to have an earlier onset and higher incidence of cancer compared to female animals [9]. 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.

This chapter will review the significant data that links 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

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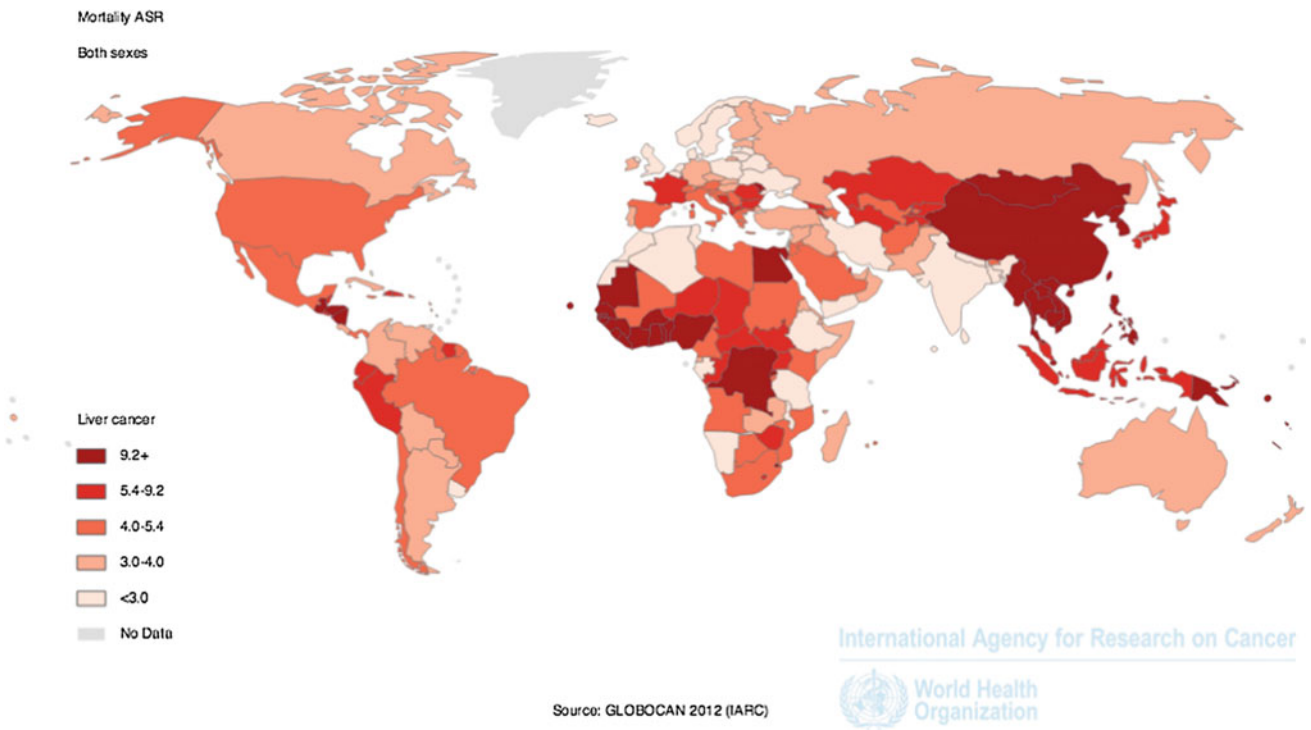

Fig. 2.1 Age-standardized mortality of liver cancer in men and women worldwide [13, 165]

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.

2.2 Molecular Biomarkers for Environmental Carcinogens

Molecular biomarkers are typically used as indicators of exposure, effect, or susceptibility for both individuals and populations. 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. The aflatoxin/liver cancer work is an exemplar of this strategy [11]. These data also help to inform the risk assessment process, where the effectiveness of regulations and policy can be tested against biological measurements of exposure and effect.

The validation of any biomarker-effect link requires parallel experimental and human studies [12]. 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. Ultimately, 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 parameters influencing efficacy.

2.3 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. 2.1 drawn from the IARC cancer data [1, 8, 13]. Since the burden of HCC is also coincident with regions where aflatoxin exposure is high, many efforts that started over 40 years ago examined this possible association [14]. 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 [15]. The commodities most often found to be contaminated by aflatoxin were common human food staples including peanuts, cottonseed, corn, and rice [16]. The requirements for aflatoxin production are relatively nonspecific since molds can produce these toxins on almost any foodstuff and the final levels in the grain product can vary from micrograms to tens of milligrams [17]. Indeed, in a case of aflatoxin-related deaths in rural villages in Kenya, daily exposures were estimated to be over 50 mg [18]. Because contamination of foodstuffs is so heterogeneous, the measurement of human exposure to aflatoxin by sampling foodstuffs or by dietary questionnaires is extremely imprecise [19]. The development and validation of specific aflatoxin biomarkers represent 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 six month intervals, were at increased risk of developing HCC [2, 20]. 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 [21–24]. The global burden of HBV infection varies widely 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 % [25]. 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 10–25 % of these individuals are likely to develop HCC [22, 26, 27]. The biology, mode of transmission, and epidemiology of this viral infection continues to be actively investigated and has been recently reviewed [25, 26, 28].

To date, the significant etiological factors associated with development of HCC in the economically developing world are infection in early life with HBV and lifetime exposure to high levels of aflatoxin B_1 (AFB_1) in the diet [29, 30]. Indeed, the multiplicative interaction between HBV and AFB_1 has been documented in two separate cohorts at high risk for HCC [31–33]. Over the past 25 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 US and Japan [34]. Detailed knowledge of the etiology of HCC has spurred many mechanistic studies to understand the pathogenesis of this nearly always fatal disease [29, 35, 36]. Fortunately, the successful development and deployment of some highly effective new drugs that cure HCV infection is a major advance and will hopefully diminish the role of this virus in liver cancer [37, 38].

A number of other environmental exposures have been epidemiologically associated with HCC [39]. Vinyl chloride exposure in occupational settings has been associated with the onset of HCC in workers and there are the classic studies associating vinyl chloride exposure with angiosarcomas in the liver [40–42]. Studies have reported a multiplicative interaction between vinyl chloride exposure in the workplace and alcohol consumption in the enhancement of HCC [43]. Finally, a synergistic interaction between vinyl chloride workplace exposure and HBV status has been reported in a cohort in Taiwan [44].

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 [45]. However, it is unclear if alcohol use in the absence of cirrhosis influences HCC development [46]. Several studies have demonstrated an increased risk of HCC up to fivefold with consumption of more than 80 g of alcohol per day or approximately 6–7 drinks per day [45]. The risk of HCC ranges from borderline significant to doubled with chronic alcohol consumption of less than 80 g/day [45]. A synergism between alcohol, and HBV and HCV infections has also been described [45, 47].

Cigarette smoke is a recognized human carcinogen; however, a causal role in HCC is unclear [48]. For example, a hospital-based case-control study in Italy found no independent effect for tobacco and HCC risk [49]. However, a composite analysis of tobacco exposure and cancer risk

consistently shows a risk for liver cancer and smoking [2, 50]. Finally, the role of hormones in the development of HCC is unclear; however, in some studies, an increase risk of HCC was observed among users of oral contraceptives [51–53]. 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.

In addition to the association of alcohol and HCC, in economically developed countries the dramatic rise in overweight and nonalcoholic fatty liver disease has also been related to increased HCC [54–56]. Of major concern for the future are the role that obesity, diabetes, and general underlying fatty liver disease will play in the development of liver cancer [57–59]. While the historic risk factors for liver cancer described above are addressed through a spectrum of prevention methods, these new etiologic factors portend an increasing trajectory in the incidence of this disease. Both therapeutic and pre-disease interventions will need to be deployed now to blunt the impact of these risk factors in the decades to come.

2.4 Methods for Biomarker Measurement

In the case of AFB_1, the measurement of the DNA and protein adducts are 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. 2.2) [14, 60, 61]. The finding that the major aflatoxin–nucleic acid adduct AFB_1-N^7-Gua excreted exclusively in urine of exposed rats spurred interest in using this metabolite as a biomarker of both exposure and risk.

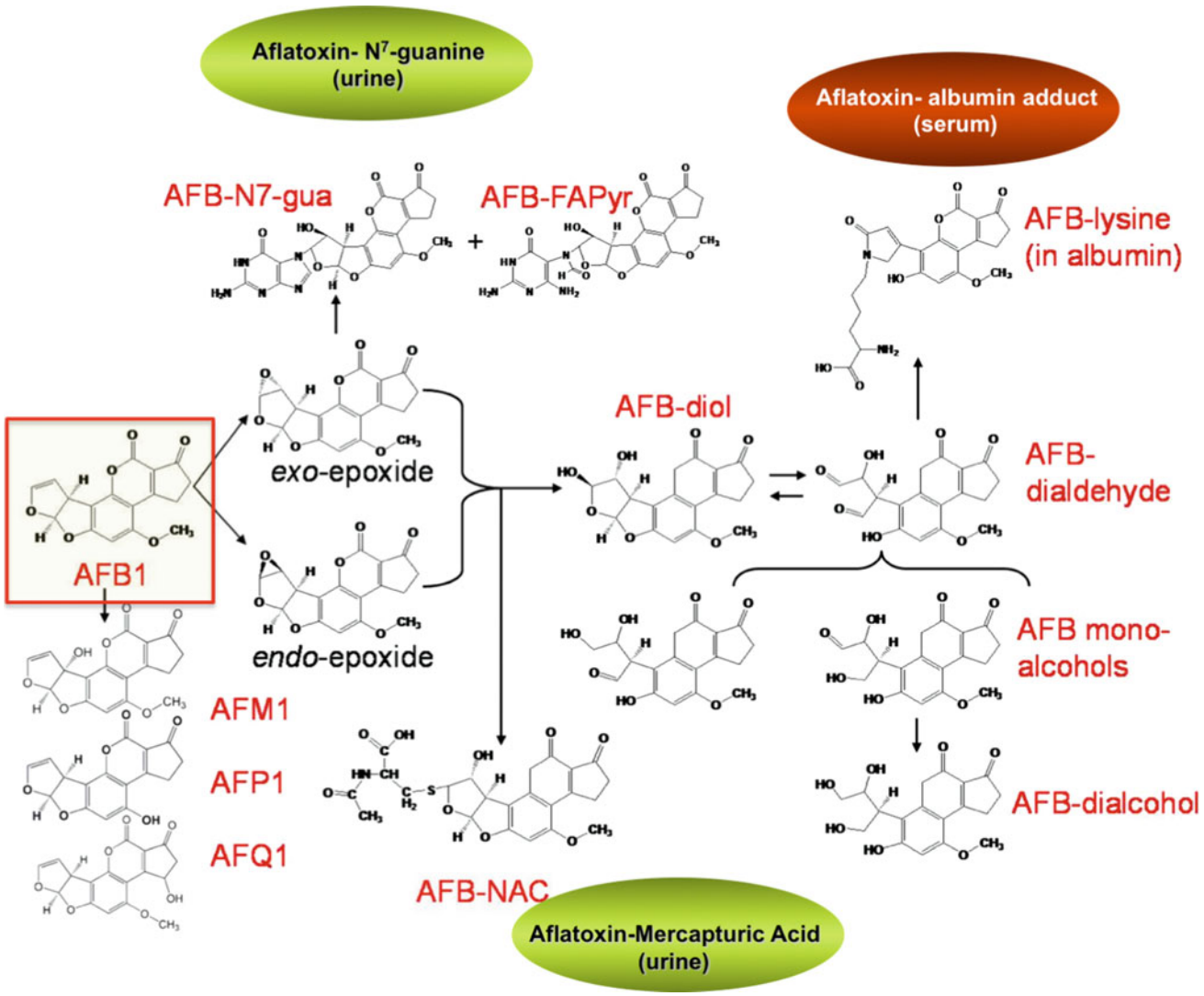

Fig. 2.2 Structures of aflatoxin biomarkers

This adduct; however, has a short half life in the body (~8 h) [62]. 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 [63].

Many different analytical methods are available for quantitation of chemical adducts in biological samples [64–66]. 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 often are more sensitive than chromatography, but immunoassays are less selective because the antibody may cross-react with multiple metabolites. An interlaboratory 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 [67].

An immunoaffinity clean-up/HPLC procedure was developed to isolate and measure aflatoxin metabolites in biological samples [68–70]. 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 [71]. 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 [72, 73]. Studies using isotope dilution mass spectrometry with liquid chromatography separation have demonstrated an increase in sensitivity of at least 1000-fold over technologies used for the detection of aflatoxin biomarkers 15 years ago [74–76]. 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 [77].

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 [78–81].

2.5 Validation of Biomarkers of Environmental Carcinogens

Over the past several decades, studies to identify effective chemoprevention strategies for aflatoxin carcinogenesis have been explored. The hypothesis was that reduction of aflatoxin–DNA and other macromolecular adduct levels by chemopreventive agents would be mechanistically related to and therefore predictive of cancer preventive efficacy. Initial data with a variety of established chemopreventive agents demonstrated that after a single dose of aflatoxin, levels of DNA adducts were reduced [82]. 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. Three months after aflatoxin treatment, it was observed that cotreatment 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 [72].

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 underrepresented the magnitude of the diminution in tumor burden [83, 84]. 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 nether 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 [85–87]. Transgenic mouse models have also been developed that generate a 100 % probability of developing HCC [88]. These transgenic mice have been used to explore the interaction of the HBV transgene with AFB_1 [89]. 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. [83] 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 [90]. Serial blood samples were collected from each animal and the integrated level of aflatoxin–albumin adducts over the exposure period decreased 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 needed.

In the most recent investigation, the synthetic oleanane triterpenoid 1-[2-cyano-3-12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), a powerful activator of Keap1-Nrf2 signaling, was found to protect against AFB_1-induced HCC. A lifetime cancer bioassay was undertaken in F344 rats dosed with AFB_1 (200 μg/kg rat/day) for 4 weeks and receiving either vehicle or CDDO-Im (three times weekly), one week prior to and throughout the exposure period. CDDO-Im completely protected (0/20) against AFB_1-induced liver cancer at 2 years of age compared to a 96 % incidence (22/23) observed in the AFB_1 group. With CDDO-Im treatment, integrated level of urinary AFB_1-N^7-guanine was significantly reduced (66 %) and aflatoxin-N-acetylcysteine, a detoxication product, was consistently elevated (300 %) after the first AFB_1 dose. The remarkable efficacy of CDDO-Im as an anticarcinogen is established even in the face of a significant aflatoxin adduct burden. Consequently, the absence of cancer requires a concept of a threshold for DNA damage for cancer development [91].

2.6 Biomarkers in Human Investigations

Extensive cross-sectional epidemiologic studies have been conducted in high-risk groups for HCC, this concept is diagramed in Fig. 2.3. 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 [92]. Further, the serology of HBV has been extensively described and developed [28]. 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 [93] 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, determined that the levels of urinary aflatoxin biomarkers showed dose-dependent relationships with aflatoxin intake. Gan et al. [94] and Wild et al. [95] also monitored levels of aflatoxin–serum albumin adducts and observed a highly significant association between intake of aflatoxin and level of adduct.

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 [96, 97]. 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 [98, 99]. 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 [100, 101]. The occurrence of this specific mutation has been mechanistically associated with AFB_1 exposure in experimental models including bacteria [102] and through demonstration that

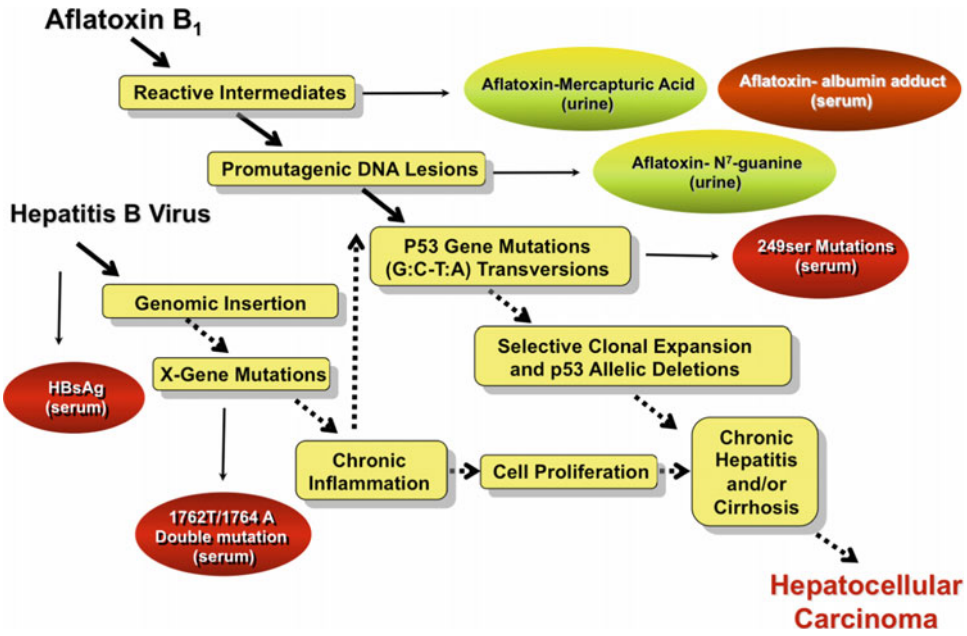

Fig. 2.3 Mechanistic-based biomarkers of aflatoxin and HBV

aflatoxin-8,9-epoxide could bind to codon 249 of *p53* in a DNA plasmid in vitro [103]. 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 [104] [105, 106]. 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 and mutagenesis data would seem to support this hypothesis [107].

Although useful, cross-sectional epidemiological studies have limited 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 [108], we needed to know whether the aflatoxin metabolites were stable over the long term. Aflatoxin–albumin adducts, as described above, in human sera were found to be stable for at least 25 years when stored at −20 °C [77].

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 [108].

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 [14].

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 that 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.

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 factors 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] [31, 32]. 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. [33] examined HCC cases and controls nested within a cohort and found that in HBV-infected people there was an adjusted odds ratio (OR) of 2.8 for detectable compared with nondetectable aflatoxin–albumin adducts and 5.5 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 [52]. 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 [32]. A small hospital-based case-control study from Northeast Thailand showed an adjusted OR of 15.2 for the presence of HBsAg among HCC patients [109]. An adjusted OR of 13.5 was reported from a case-control study in The Gambia [25]. 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 [110]. A similar prospective study from Taiwan found men positive for HBsAg were 223 times more likely to develop HCC than men HBsAg negative [23].

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 [111, 112]. 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 [113–115]. 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 [116]. The increasing occurrence of these mutations have been also associated with the increasing severity of the HBV infection and cirrhosis [114, 115]. This acquired mutation following HBV integration into hepatocytes was originally characterized in HBV e antigen negative people [117]. 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 [118–120]. 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 [121].

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 [121–123]. 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 [111, 112]. In cellular studies, the 1762^{T}/1764^{A} double mutation increased the replication of the viral genome twofold and in the case of some of rarer triple mutations, an eightfold increase in genome replication was found [111, 123]. Recent data have also shown that there is a sequential accumulation of these mutations in people during the course of the progression to cancer [124].

Recently, a matched case-control investigation of 345 men who died of HCC and 625 controls were nested within a cohort of male HBsAg carriers from Qidong, China. Matched preserving odds ratios (ORs) were used as a measure of association and 95 % confidence intervals (CIs) as a measure of precision. A total of 278 (81 %) of the cases were positive for the HBV 1762^{T}/1764^{A} mutation compared with 250 (40 %) of the controls. The matched preserving OR of 6.72 (95 % CI: 4.66–9.68) strongly indicated that cases were significantly more probably than controls to have the mutation. Plasma levels of DNA harboring the HBV mutation were on average 15-fold higher in cases compared with controls ($P < 0.001$). Most strikingly, the level of the mutation in the 20 controls which later developed and died of HCC were on average 274-fold higher than controls which did not develop HCC. Thus, within this cohort of HBsAg carriers at high risk of developing HCC, individuals positive for the HBV 1762^{T}/1764^{A} mutation at enrollment were substantially more probably to subsequently develop HCC, with a higher concentration of the mutation in plasma enhancing predisposition for cancer development [125].

2.7 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 [126]. 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 reported [127]. This study assessed 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 maybe an effective means to reduce HCC burden, especially in areas where single foodstuffs such a groundnuts are major components of the diet.

Chemoprevention is another strategy for the secondary prevention of cancer. This approach entails the use of drugs, dietary supplements or functional foods to retard, block, or reverse the carcinogenic process. These strategies serve to alter cell fate, by either preventing cells from acquiring carcinogenic genetic damage or by impeding proliferation of preneoplastic cells or, alternatively, accelerating their apoptosis. One successful strategy for cancer chemoprevention is modulation of drug-metabolizing enzymes, leading to facilitated inactivation or elimination of endogenous and environmental carcinogens. Inducers of conjugating enzymes such as dithiolethiones and sulforaphane inhibit tumorigenesis of environmental carcinogens in various animal models [83, 128]. Increasing lines of evidence show that the Keap1-Nrf2 complex is a key molecular target of these chemopreventive enzyme inducers. The transcription factor Nrf2 is a member of the basic leucine-zipper NF-E2 family and interacts with the antioxidant response element (ARE) in the promoter region of detoxifying enzymes. A cytoplasmic actin binding protein, Keap1, is an inhibitor of Nrf2 that sequesters it in the cytoplasm and facilitates its ubiquitination and subsequent degradation. Inducers disrupt this process, allowing Nrf2 to accumulate and translocate to the nucleus [129]. Experimental disruption of the *Nrf2* gene in mice leads to enhanced sensitivity to carcinogens and the loss of chemopreventive efficacy by inducers [130, 131].

1,2-Dithiole-3-thiones were reported in the 1950s to be constituents of cruciferous vegetables in Czechoslovakia [132], although a more recent study failed to find the unsubstituted 3*H*-1,2-dithiole-3-thione in cabbage in the United States [133]. Oltipraz, a substituted 1,2-dithiole-3-thione, was originally developed by the pharmaceutical

industry as a possible treatment for schistosomiasis and was extensively evaluated in clinical trials in the early 1980s. In extensive preclinical evaluation by the National Cancer Institute and others, oltipraz was found to be effective as an anticarcinogen in nearly a score of animal models [134].

Aflatoxin biomarkers (Fig. 2.2) were used as intermediate endpoints in a Phase IIa liver cancer chemoprevention trial of oltipraz in Qidong, People's Republic of China [135, 136]. This was a placebo-controlled, double-blind study in which participants were randomized to receive placebo or 125 mg oltipraz daily or 500 mg oltipraz weekly. Urinary aflatoxin M_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 aflatoxin M_1 levels in the 125-mg group compared with placebo. This effect was thought to be due to inhibition of cytochrome P450 1A2 activity. Median levels of aflatoxin–mercapturic acid (a glutathione conjugate derivative) were elevated six-fold in the 125-mg group, but were unchanged in the 500-mg group. Increased formation of aflatoxin–mercapturic acid reflects induction of aflatoxin conjugation through the actions of glutathione-S-transferases (GSTs). The apparent lack of induction in the 500-mg group was thought to reflect masking caused by diminished aflatoxin-8,9-epoxide formation for conjugation through the inhibition of CYPlA2 seen in this group. This initial study demonstrated for the first time that aflatoxin biomarkers could be modulated in humans in a manner that would predict decreased disease risk.

Although the oltipraz clinical trial demonstrated the proof of principle for increasing pathways leading to aflatoxin detoxication in humans, the practicality of using a drug-based method for prevention in the economically developing world is limited. Not only is there a potential for adverse health effects from any long-term exposure to a drug, but also the expense of this type of intervention may make the intervention cost-prohibitive for these populations. There may also be culture-based aversion to the use of drugs. Fortunately, oltipraz is not the only agent that affects enzyme changes through the Nrf2-Keap1 pathway. Sulforaphane has been extensively examined for its chemopreventive properties and is a potent activator of the Nrf2-Keap1 pathway leading to increased expression of carcinogen detoxifying enzymes [131, 137]. Many foods have high levels of these enzyme inducers [138, 139]. In a recent chemoprevention trial in humans, a beverage formed from hot water infusions of 3-day-old broccoli sprouts, containing defined concentrations of glucosinolates (a stable precursor of the anticarcinogen sulforaphane), was evaluated for its ability to alter the metabolic disposition of aflatoxin. In this study, 200 healthy adults drank infusions containing either 400 or <3 μmole glucoraphanin nightly for 2 weeks. Urinary levels of aflatoxin-N^7-guanine were similar in individuals in the two intervention arms. However, measurement of urinary levels of dithiocarbamates (sulforaphane metabolites) indicated striking interindividual differences in bioavailability. This outcome may reflect individual differences in the rates of hydrolysis of glucoraphanin to sulforaphane by the intestinal microflora of the study participants. Accounting for this variability, a significant inverse association was observed for excretion of dithiocarbamates and aflatoxin-N^7-guanine adducts in individuals receiving broccoli sprout glucosinolates [140]. This preliminary study illustrates the potential use of an inexpensive, easily implemented, food-based method for secondary prevention in a population at high risk for aflatoxin exposures. A follow-up intervention designed to minimize the interindividual variability in the pharmacokinetics of the glucoraphanin precursor is currently in progress.

Many studies have demonstrated that green tea polyphenols (GTP) inhibit various chemically induced cancers in experimental animals, and epidemiological studies also point to the potential benefit of these compounds [141, 142]. Qin et al. [143] studied the effects of GTP in drinking water for two or four weeks to protect against the development of AFB_1-induced hepatocarcinogenesis in the rat. Results revealed that aflatoxin–DNA binding in the liver was significantly (20–30 %) inhibited in animals pretreated with green tea and that the burden of preneoplastic lesions was also significantly inhibited by 60–70 %. The experimental data on GTP provided the impetus to translate this strategy to human clinical trials. In an initial study in an aflatoxin-exposed high-risk group in Guangxi, People's Republic of China, the effects of GTP was assessed by analysis of blood and urine samples collected from a randomized, double-blinded, placebo-controlled Phase IIa chemoprevention trial [144]. Blood serum of all participants contained aflatoxin–albumin adducts at the outset. They were then required to ingest capsules containing GTP at doses of 500 or 1000 mg, or a placebo daily for 3 months. Analyses were done on blood and urine samples collected during this period to evaluate the efficacy of GTPs in modulating aflatoxin biomarkers [145]. Levels of albumin adducts at baseline were comparable for all three dose groups and no significant differences were observed in adduct levels in the placebo group over the 3 month period. However, reductions in albumin adduct levels were observed in both groups receiving GTPs over the 3 month intervention period. An analysis using a mixed-effects model indicated that the reduction in aflatoxin–albumin adduct levels over time was dose- and time-dependent. Reductions in median aflatoxin M_1 levels, as compared with the placebo, were found in both GTP groups at 3 months of the intervention, while significant elevations in median aflatoxin–mercapturic acid levels were observed in both GTP groups compared with the placebo group at 1 and 3 months of intervention. These results

indicate that GTPs effectively modulate aflatoxin metabolism and metabolic activation, as had been previously observed with oltipraz in Qidong.

2.8 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 [146]. 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 [147]. 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 [148–151]. 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 [79, 152, 153]. The process by which tumor DNA is released into circulating blood is unclear but may result from accelerated necrosis, apoptosis, or other processes [154]. 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 [155]. In a seminal report, Kirk et al. [156] 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 were 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. [79], 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, six 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 1638 high-risk individuals in Qidong, PRC., that have been followed since 1992 [157]. 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 pre-diagnosis 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 one 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 HCC cases, 42 cirrhotic patients, and 131 nonliver diseased control subjects, all from highly aflatoxin-exposed regions of The Gambia [81]. The HCC cases had higher median plasma concentrations of the p53 mutation (2800 copies/mL; interquartile range: 500–11,000) compared with either cirrhotic (500 copies/mL; interquartile range: 500–2600) or control subjects (500 copies/mL; interquartile range: 500–2000. Levels of >10,000 copies of p 53 codon 249 mutation/mL plasma were also significantly associated with the diagnosis of HCC (OR, 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.

2.9 Summary

HCC is a slowly developing process involving progressive genetic insults and their resulting genomic changes. The advances in modern DNA sequencing technologies have been used on a wide number of human liver cancers with a range of etiological factors that reveal a very complete picture of driver and passenger mutational changes in these tumors [158–160]. These data will hopefully form a foundation for new therapies and early detection screening methods. Further, as these sequencing methods become extended to characterize microRNAs and proteomic methods help characterize the molecular phenotype of liver cancers, these collective data will help define the preclinical period of tumor development. This will be very valuable for our mechanistic understanding of HCC up to 30 years after chronic infection with HBV, HCV and/or aflatoxin exposure prior to clinical diagnosis. These studies may also reveal insights into chronic hepatitis and cirrhosis since 70–75 % of all HCC is accompanied by cirrhosis [113, 158].

The molecular epidemiology investigations of aflatoxin, HBV, and HCC probably represent one of the most

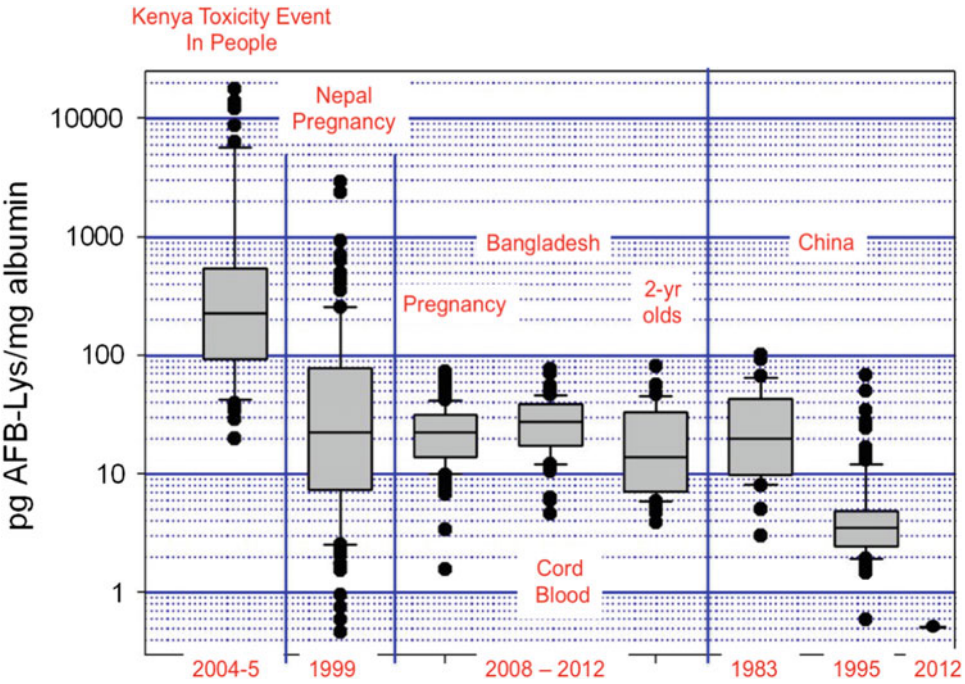

Fig. 2.4 Range of aflatoxin exposure in different populations [162]

extensive data sets in the field of environmental carcinogenesis and this work serves as a template for future studies of the role of other environmental agents in human diseases with chronic, multifactorial etiologies. 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.

Recent data utilizing the cancer registry in Qidong, China has provided some very exciting insights into the role of aflatoxin in liver cancer. Utilizing the availability of serum samples collected over a 20-year period, aflatoxin exposure patterns have been documented. In China, major agricultural reforms in the 1980s led to diminished maize consumption, a major source of aflatoxin contamination. The population-based cancer registry in Qidong, China has documented a more than 50 % reduction in HCC mortality rates occurring across birth cohorts from the 1960s to the 1980s for Qidongese less than 35 years of age although all were born before universal vaccination of newborns. Median levels of the aflatoxin biomarker decreased from 19.3 pg/mg albumin in 1989 to undetectable (<0.5 pg/mg) by 2009. A population attributable benefit of 65 % for reduced PLC mortality was estimated from a government-facilitated switch of dietary staple from maize to rice; 83 % of this benefit was in those infected with HBV. Food policy reforms in China thus resulted in a dramatic decrease in aflatoxin exposure, which, independent of HBV vaccination, reduced liver cancer risk. The extensive HBV vaccine coverage now in place augurs even greater risk reductions in the future [161].

Finally, in an attempt to place the extent of global exposures to aflatoxin across different populations, with varying health endpoints, we have determined the aflatoxin–albumin adduct levels shown in Fig. 2.4. These samples were from studies in Nepal, Bangladesh, Kenya during an acute toxic event and China [67, 161–163]. We note that a 1 μg per day exposure results in a 0.7 pg/mg albumin adduct level and this increases linearly using adduct formation data gleaned from human exposure studies [164]. Thus these findings provide for the first time a guidepost for relating daily exposure levels to acute and chronic disease outcomes and using biomarkers the efficacy of policy and regulation can be objectively measured.

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Molecular Mechanisms of Hepatocellular Carcinoma

3

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Contents

3.1 Introduction

Hepatocellular carcinoma is a highly heterogeneous disease for different reasons. First, there are multiple and highly variable etiological factors including viruses with a DNA (HBV) or RNA (HCV) genome, chemicals (alcohol and aflatoxins), and inborn and acquired metabolic diseases. Second, these cancers might originate either from mature hepatocytes or from progenitor cells. Third, like other cancers, HCC undergoes a dynamic process changing morphology and molecular features as it advances. Therefore, molecular mechanisms of hepatocellular carcinogenesis may vary depending on different factors and this is probably why a large set of mechanisms have been associated with these tumors. Among many different mechanisms described, we review here those that we believe are the most prominent ones including loss of cell cycle control, escape from senescence control, resistance to cell death, phenotypic plasticity, motility, invasion, and metastasis.

3.2 2-Loss of Cell Cycle Control

One of the common features observed in all tumorigenic cells is the loss of cell cycle control, which leads to an elevated proliferative capacity, hyperplasia, and eventually tumor formation [1]. The alterations of the core cell cycle machinery genes are not among the frequently observed driver mutations in hepatocellular carcinoma [2]. Nevertheless, loss of cell cycle control due to alterations in molecular pathways that either upregulate genes or activate proteins promoting cell cycle entry and progression; or downregulate genes or inhibit proteins regulating this process, are common events in HCC [3].

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3.2.1 Cell Cycle Regulation in Hepatocytes

Hepatocytes of a normal liver predominantly exist in the quiescent phase (G0) of the cell cycle and do not routinely divide. However, they retain the capacity to proliferate and following the reception of mitogenic signals they are able to enter the cell cycle and execute the sequence of events in order to complete the cell division. The best example demonstrating the proliferative potential of hepatocytes is the surgical removal of 70 % of the liver mass by partial hepatectomy upon which the remaining cells do compensate the lost tissue mass by completing roughly two rounds of divisions per cell [4, 5]. The general molecular machinery and mechanisms regulating the progression of hepatocytes as well as other proliferating cells through different phases of the cell cycle are well conserved [6].

Progress through the different phases of the eukaryotic cell cycle (G0/G1, S, G2, and M) is regulated by the concerted actions of cyclin-dependent kinases (Cdk) and their partner/activator cyclins. Each of the Cdk/cyclin complexes operates during a specific phase of the cell cycle and is responsible for the execution of certain events such as initiation of DNA replication, chromosome condensation, etc. Nevertheless, there is a wide degree of compensation between Cdk/cyclin complexes [7], with only the Cdk1/cyclin B1 complexes being essential for the completion of the mitotic cell cycle [8].

In quiescent hepatocytes, entry into the cell cycle is initially promoted by cytokines and growth factors that result in expression of cyclin D1 gene [9–11]. De novo-expressed cyclin D1 forms complexes with Cdk4 and Cdk6, which phosphorylate the retinoblastoma protein (pRb) [12] causing a conformational change. This in turn results in the release of E2F transcription factors and transcription of cell cycle genes such as cyclins E, A and B and Cdk1. Resulting Cdk2/cyclin E complexes further phosphorylate and inactivate the pRb protein, promoting the cell cycle progression [13, 14]. Cdk2/cyclin E complexes initiate, and Cdk2/cyclin A complexes maintain and complete DNA replication. Association of Cdk1, first with cyclin A and then cyclin B results in phosphorylation of a vast number of substrates and entry into mitosis [6].

Several regulatory pathways prevent quiescent hepatocytes from entering cell cycle and proliferating freely. As mentioned above, pocket proteins including pRb, p107, and p130 bind to and sequester E2F transcription factors in order to repress its transcriptional activity directed at cell cycle and proliferation genes [15, 16]. Ink4 family inhibitors (p15, p16, p18, and p19) prevent entry into the cell cycle by binding to Cdk4/6 kinases and preventing complex formation with cyclin D [17]. On the other hand, the Cip/Kip family inhibitory proteins p21, p27, and p57 inhibit cell cycle progression by binding and inactivating all Cdk/cyclin complexes [18].

3.2.2 Cell Cycle Regulatory Pathway Aberrations in HCC

3.2.2.1 Cyclin D1

Comparison of gene expression profiles of HCC samples and surrounding non-tumor liver tissues has identified a "proliferation cluster" that includes cell cycles genes including Cdks and cyclins displaying an increased expression levels [19, 20]. The reason behind this observation is not specific genomic alterations that lead to increased expression of these genes, but rather the presence of an abundant population of highly proliferative tumor cells that express cell cycle genes.

Nevertheless, chromosomal loci harboring the cyclin D1 and cyclin E1 genes are amplified in 10–20 % of HCC [21–23]. Other causes of the high-level expression of the cyclin D1 protein in many HCC samples are the mitogenic pathways that control its transcription, translation, and degradation. Several signaling pathways that are altered during HCC development, such as Ras/MAPK, Wnt/β-catenin, result in increased expression of cyclin D1 [24, 25]. Another potential reason for the elevated cyclin D1 expression could be downregulation of microRNAs miR-195 and miR-520 in some HCC samples, as both of them target Cyclin D1 expression when overexpressed in HCC cell lines [26, 27]. Although mutant forms of β-catenin activate transcription from the cyclin D1 leading to increased expression in colon cancer cells [28], there is no consistent positive correlation between cyclin D1 and β-catenin protein levels in HCC [29, 30]. Chronic overexpression of cyclin D1 gene in transgenic mice results in development hepatocellular carcinoma in 30 % of the animals, however it takes 17 months [31]. This suggests that genomic alterations increasing cyclin D1 expression could in principle trigger HCC development.

3.2.2.2 p16 (CDKN2A)

As the p16/pRb pathway control entry into cell cycle, any aberrations that reduce the expression levels of these genes or interfere with the protein functions are potentially tumorigenic. Aberrant expression of the pRb is quite common in HCC [32], with half of the tumors showing no expression at all [33]. Alterations in the pRb pathway are covered in detail in this chapter (see Sect. 3.3 escape from senescence control) and therefore will not be further elaborated here.

Expression of p16 (CDKN2A) is often down-regulated via hypermethylation of its mutual promoter with p14 [34,

35]. Hepatitis B virus X protein increases the expression of DNA methyl transferases (DNMT1 and DNMT3A) in HCC resulting in hypermethylation of the p16 gene locus and leading decreased gene expression [36, 37]. Germ-line or de novo mutations that prevent p16-Cdk4/6 interactions also exist but are much less frequent [38, 39].

3.2.2.3 CIP/KIP Family: p21 and p27

CIP/KIP family member proteins p21 and p27 inhibit Cdk/cyclin complex activity and therefore act as brakes on cell cycle progression. Their expression levels are often found to be decreased in human HCC samples [40–42], however this is not a general rule as contradictory studies that found increased expression of p21 and p27 also exist in literature [41, 43].

Loss of p53 function due to mutations or deletions results in absence of p21 transcription in HCC [44]. In addition to p53, HCV core protein can alter the expression level of p21 in a p53 dependent manner, with its immature and mature forms having positive and negative effects, respectively, on p21 expression [45, 46]. Another potential way of regulation is miR-345 expression through mature HCV core protein as it has been shown to result in downregulation of p21 expression in human hepatoma cells [47].

p27 is another regulator of cell cycle progression whose expression level can be decreased via different mechanisms in HCC [48, 49]. p27 deficient transgenic mouse models display an increased hepatocyte density in their livers in parallel with hyperplasia in multiple organs ([50, 51]; Nakayama et al. 1996), however they do not spontaneously develop HCC unless their livers are chronically injured [52]. Loss of p27 function in hepatocellular cancers generally occurs either posttranscriptionally or posttranslationally. p27 mRNA is targeted for destruction by miR-221, whose expression is increased in majority of HCC samples [53]. Posttranslational regulation of p27 function can be either via ubiquitin-mediated degradation or cytosolic sequestration through phosphorylation [54]. The former is achieved by Skp2, which targets p27 as well as p21 and p57 for ubiquitin-mediated degradation; and is expressed at higher levels in HCC due to deregulation of its transcription [55, 56]. Although phosphorylation of p27 by Cdk2 targets it for ubiquitin-mediated degradation, phosphorylation at Thr157 by PKB/Akt kinase prevents its nuclear localization and causes cytosolic sequestration [57] and worsens the prognosis in HCC [58].

3.3 Escape from Senescence Control

Cellular senescence is a form of irreversible growth arrest associated with well-defined morphological changes in cell culture [59]. It has long time been considered as a cellular mechanism that limits the number of cell divisions [or population doublings (PDs)] in response to progressive telomere shortening that occurs in proliferating normal somatic cells because they lack efficient telomerase activity. This form of senescence is now recognized as replicative (or telomere-dependent) senescence. Telomere-dependent senescence occurs as a result of progressive telomere shortening in cell culture [60]. Telomeres are regions which contain repetitive DNA elements with variable length (5–20 kb) and telomere-binding proteins at the ends of chromosomes. Telomeric DNA has a structure called “t-loop” formed as a result of invasion of the single-stranded G-rich sequence into the double-stranded telomeric tract. Telomeres, with telomere-binding proteins, prevent genomic instability and the loss of essential genetic information by “capping” chromosome ends. They are also indispensable for proper recombination and chromosomal segregation during cell division. Telomeres become shorter in every cell division in somatic cells, because of the inability of replication complex to copy the ends of linear DNA which makes them a “cell cycle counter” for a cell [61]. Telomeres are added to the end of chromosomes with a complex containing an RNA template called telomerase RNA component (TERC) and a reverse transcriptase called telomere reverse transcriptase (TERT) enzyme [62]. Most somatic cells lack telomerase activity because the expression of TERT is repressed, in contrast to TERC. The lack of sufficient TERT expression in somatic cells is the main cause of telomere shortening during cell replication [62].

Senescence-like changes can be induced in young proliferating cells in response to different forms of cellular stress such as DNA damage and oncogenic stimuli, in the absence of telomere shortening [60]. Many forms of senescence-inducing stresses have in common the ability to affect cellular DNA integrity. Therefore, one of the main purposes of senescence response appears to block the proliferation of cells with damaged DNA.

The major pathways leading to characteristic morphological changes in senescent cells are still ill-defined, but the main pathways controlling their proliferation status are known. Proliferation arrest in senescent cells is often accompanied with accumulation of cells at G1. As stated earlier, the transition from G1 to S phase requires the release of E2F factors from their inhibitory partner pRb. The senescence arrest is mediated by inhibition of pRb phosphorylation by p16 and p21 acting on cyclin-dependent kinases. The p16 is transcribed from the INK4a/ARF locus which also encodes transcripts for another protein named p14ARF [60]. p16-dependent senescence arrest appears to be more important for telomere-dependent senescence, whereas p21-dependent arrest is involved in both telomere-dependent and telomere-independent senescence arrest. The accumulation of p16 in telomere-dependent

senescence was linked to BMI1 component of polycomb complex [63, 64]. Recently, the ability of the BMI1 to repress the INK4a/ARF locus was shown to depend on EZH2-containing polycomb-repressive Complex 2 [63]. EZH2 is downregulated in senescent cells, while it is overexpressed in tumors, including HCC [65, 66]. p21-dependent pathway mediates p53-dependent, but also some p53-independent signals for senescence arrest.

The p53 protein is the major player of senescence arrest as it may be upregulated by both telomere-dependent and telomere-independent pathways. The p14ARF protein which can be induced by the derepression of INK4/ARF locus [63] or during oncogene-induced senescence leads to p53 accumulation via inhibition of MDM2 protein [60]. p53 is also induced by DNA damage, independently from p14ARF by MDM2-dependent and MDM2-independent mechanisms via DNA damage checkpoint proteins [67, 60]. p53 accumulation in senescent cells induces cell cycle arrest via its transcriptional target p21. p21-dependent pathway also appears to play a role in p53-independent senescence induction, as shown in cancer cells [68, 69].

Both p16 and p21-dependent senescence arrests must play major anticancer functions in mammalian cells, since many components of this senescence pathways undergo genetic and epigenetic alterations in cancer cells which are quite often resistant to senescence arrest [60].

3.3.1 Replicative Senescence of Hepatocytes

Hepatocytes in the adult liver are renewed approximately once a year, as estimated by telomere loss which is 50–120 bp per year in healthy individuals [70]. However, the liver has an extremely powerful regenerative capacity, as demonstrated experimentally in rodents, and as observed in patients with chronic liver diseases [71]. This regenerative capacity is due mostly to the ability of mature hepatocytes to proliferate in response to a diminution of total liver mass either experimentally, or following exposure to viral and nonviral hepatotoxic agents. In addition, adult liver seems to harbor hepatocyte-progenitor cells that are able to restore liver hepatocyte populations [72]. However, hepatocytes, like any other somatic cells, do not have unlimited replicative capacity, due to the lack of telomerase activity that is needed to avoid telomere shortening during successive cell divisions. This is best exemplified by decreasing hepatocyte proliferation in liver cirrhosis stage of chronic liver diseases [73], providing in vivo evidence for exhaustion of hepatocyte proliferation capacity.

As stated earlier, limited proliferative capacity of somatic cells is controlled by replicative senescence [74]. However, our knowledge of hepatocyte replicative senescence is highly limited. In contrast to in vivo conditions, mature hepatocytes are extremely resistant to cell proliferation in cell culture. Usually, more than 99.9 % of adult liver hepatocytes do not divide in cell culture and can only be maintained in culture for a few weeks. A small progenitor-type cell population (so-called small hepatocytes) has been shown to proliferate in vitro, but they usually stop growing at passages 5–7, with an ill-defined senescence-like phenotype [72]. Fetal hepatocytes display better proliferation capacity in culture. A few studies have shown that these fetal cells enter replicative senescence, as shown by senescence-associated β-galactosidase assay (SABG) at population doubling (PD) 30–35 [75]. This is accompanied by progressive shortening of telomeres down to ~6 kbp, as these cells like adult hepatocytes lack telomerase activity. Fetal hepatocytes can be immortalized by stable expression of TERT [75].

3.3.2 In Vivo Senescence in Liver Tissue

The liver is one of the rare tissues where in vivo telomere shortening and replicative senescence have been convincingly and independently demonstrated by different investigators [76]. Telomere shortening in normal human liver and its stepwise exaggeration in chronic hepatitis, cirrhosis, and HCC has been described more than a decade ago [77], and confirmed by many studies [78, 79]. Both p21 and p16 expression was found to be high in cirrhosis, as compared to normal liver, as well as malignant lesions [80], suggesting that these major senescence-inducing proteins accumulate in cirrhotic liver, in support of the hypothesis that cirrhosis represents a stage of in vivo hepatocyte senescence.

The relevance of replicative senescence to liver tissue aging has been demonstrated experimentally using TERC-deficient mice. Late generation mice with TERC-deficiency display critically shortened telomeres in liver whose regenerative response to partial hepatectomy is impaired. A subpopulation of telomere-shortened hepatic cells display impaired proliferative capacity that is associated with SABG activity [81]. In addition, experimentally induced telomere dysfunction was shown to induce p53-dependent senescence in mice expressing a dominant-negative mutant TRF2 protein [82].

3.3.3 p53 and Retinoblastoma Pathways in Hepatocellular Carcinoma

p53 and retinoblastoma (RB) pathways play a crucial role in senescence arrest that appears to mark cirrhosis stage in chronic liver diseases. HCC rarely develops in liver tissues in the absence of chronic liver disease. More than 80 % of these cancers are observed in patients with cirrhosis. As the

appearance of proliferating malignant cells from this senescence stage requires the bypass of senescence, the status of both p53 and RB pathways in HCC is of great importance in terms of molecular aspects of hepatocellular carcinogenesis.

Tumor suppressor gene TP53 (p53) is mutated or inactivated in various types of human cancers with a mutation frequency of more than 50 %, independent of tissue origin and etiology. There are five hotspots scattered in DNA-binding domain (amino acid residues 175, 248, 249, 273, and 282) of p53 protein frequently affected by mutations [83]. HCC is one of the major tumors displaying frequent p53 mutations [84]. The overall p53 mutation frequency in HCC is around 30 %. However, both the frequency and the spectrum of p53 mutations show great variations between tumors from different geographical areas in the World. This appears to reflect the relative contribution of different etiologic factors showing high variation among different continents. Aflatoxin exposure is confined to some parts of Africa and East Asia, where hepatitis B is also prevalent. In other continents, hepatitis B, hepatitis C, and alcoholism are more prominent factors for hepatocellular carcinogenesis. The highest rates of p53 mutations are observed in aflatoxin-contaminated areas of Africa and East Asia which fluctuate between 50 and 80 % of reported cases. This high frequency is due almost exclusively to an HCC specific hotspot mutation at codon 249 (AGG → AGT) leading to an arginine to serine substitution (R249S). As G → T transversions are major mutation products of aflatoxins in experimental systems, this led to a hypothesis that the codon 249 mutation of p53 in HCC is induced by aflatoxins [84]. The detection of the same mutation in normal-appearing liver tissues in people at high risk for aflatoxin exposure supports the hypothesis that aflatoxins have a causative and probably early role in HCC. In countries in which aflatoxin is not a known etiological factor, the rate of p53 mutations is low and the spectrum of mutations is scattered within the DNA-binding domain, codon 249 mutations being detected only occasionally [84]. Several studies reported that p53 could be inhibited by hepatitis viral proteins also [85–87].

Senescence arrest mediated by p53 involves upstream and downstream proteins of p53 pathway. Among upstream molecules, Mdm2 promoter SNP309 was found to be a risk factor for HCC [88]. Promoter methylation of p14ARF was identified in 19 % of HCCs associated with HBV and in 39 % of HCCs associated with alcohol, respectively [89]. The expression of p21 gene which is a common intermediate between p53 and RB pathways was reported to be downregulated in 73 % of HCCs [90]. Similarly, Weinmann et al. reported a decreased expression of p21 in HCC lesions, as compared to cirrhotic hepatocytes [79]. However, the biological significance of these observations is presently unknown.

Two main players of RB pathway involved in senescence arrest are pRb and p16. Mutations of these genes are uncommon in HCC. The loss of heterozygosity was reported to occur in retinoblastoma gene (RB1), but its mutational inactivation in HCC has not been demonstrated. On the other hand, the gankyrin, which promotes proteosomal degradation of pRb protein was found to be overexpressed in HCC [91]. The only consistent mutation affecting the RB pathway in HCC is the homozygous deletion of INK4a/ARF locus, but this occurs at a frequency of less than 10 % [39, 92]. On the other hand, epigenetic silencing of p16 gene by promoter methylation is highly frequent in HCC (see Ozturk [84]). In a combined study [89], retinoblastoma pathway alterations (p16, p15INK4b or RB1 genes) were present in 83 % of HCCs, whereas p53 pathway alterations (p53 or p14ARF genes) were detected in only 31 % of these tumors. Alterations in both RB and p53 pathways were present in 30 % of HCCs. Thus, it appears that either the RB and/or the p53 pathway is affected in the majority of HCCs, and that both pathways are affected in at least one third of these tumors.

3.3.4 Telomerase Reactivation and Immortality in Hepatocellular Carcinoma

The cause–effect relationship between cirrhosis and HCC is so evident that the cirrhosis is considered as a common etiological factor of these cancers. As cirrhosis is dominated by telomere-dependent senescence arrest in hepatocytes, HCC development requires a bypass by reactivation of TERT, the enzyme missing in normal hepatocytes. Therefore, it is not surprising that strong telomerase activity was detected in 80–90 % of HCCs [93–95]. A recent study provided experimental evidence for the presence of thousands of senescence- and immortality-associated genes with differential expression between liver cirrhosis and HCC [96]. As expected, genes overexpressed in cirrhosis are those that are induced during senescence, whereas many genes overexpressed in HCC are induced in immortal or immortalized cells. Thus, there is ample evidence that a phenotypic switch from senescence to cellular immortality must occur during the initiation of HCC, in particular those observed in cirrhotic patients [96].

The mechanisms of hepatocellular immortalization are not completely understood. It is clear that one key mechanism involved in this process is the reactivation of TERT in order to prevent telomere shortening during successive proliferation cycles. The TERT reactivation in cancer cells remained as an enigma until very recently. Initial findings

with HCC showed that HBV DNA can integrate into the TERT gene and found as one of the paths to increase telomere length in these cancers [97–99]. However, this occurs quite rarely, and many HCCs are not related to HBV. Recently, many groups reported the presence of two frequent mutations in TERT promoter region in different tumors, including HCC [100–105]. These promoter mutations are claimed to upregulate the TERT transcription by creating a binding site for ETS (E-twenty six) [101] and ternary complex factor (TCF) transcription factors [100]. TERT promoter mutation frequencies of the reported for HCC varied between 24 and 59 % depending on geographical locations [102, 106, 107]. We recently reported that the TERT mutations (C228T and C250T) were more frequent in African HCCs, when compared to non-African tumors. We also found a weak inverse correlation between TERT promoter mutations and MDM2 SNP 309 TG polymorphism [106]. Overall, these studies revealed that one of the most frequently mutated genes in HCC is TERT gene.

The timing of TERT mutations during hepatocellular carcinogenesis was studied in detail by Nault et al. [107]. No TERT mutation was found in cirrhosis and hepatocellular adenoma. However, TERT mutation was the earliest genetic change observed in cirrhotic macronodules with or without dysplasia. A similar study further indicated that TERT mutation frequencies displayed a stepwise increase during hepatocellular carcinogenesis, i.e., 6 % of low grade dysplastic nodules, 9 % of high grade dysplastic nodules, 61 % of early HCCs, and 42 % of small and progressed HCC. Thus, the TERT mutation is likely to mark the beginning of an overtly malignant state (i.e., early HCC) during hepatocellular carcinogenesis [108].

3.3.5 Experimental Induction of Senescence in Hepatocellular Carcinoma

Critical senescence bypassing events that are frequently observed in HCC strongly suggest that experimentally induced senescence arrest my serve as a powerful strategy to revert HCC malignancy. Treatment of HCC cell lines with 5-aza-20-deoxycytidine induced the expression of p16, hypophosphorylation of pRb and G1 arrest associated with positive SABG staining [109]. Xue et al. [110] expressed H-ras oncogene and suppressed endogenous p53 expression in mouse hepatoblasts which produced massive HCCs upon implantation into livers of athymic mice. However, these tumors regressed rapidly upon restoration of p53 expression. Tumor regression was associated with differentiation and massive senescence arrest followed by immune-mediated clearance of senescent cells. Overexpression of c-Myc in liver-induced HCC that reversed completely upon c-Myc inactivation. Tumor cells lacking c-Myc activity displayed differentiation into mature hepatocytes and biliary cells, as well as senescence response [111], and regressed progressively by massive apoptosis [112].

We tested whether transforming growth factor-b (TGF-β) could serve as a potential senescence inducer in HCC. Five HCC cell lines with intact TGF-β signaling (Smad-targeted gene activation by TGF-β) displayed also a strong senescence response which was p53- and p16-independent. Senescence was associated with Nox4-mediated ROS accumulation and was both p21- and p15-dependent. Moreover, when induced in vivo, TGF-β-dependent senescence was a strong inhibitor of HCC tumor growth [113]. Thus, tumor regression by senescence induction appears to be an efficient anti-HCC therapy method, at least experimentally.

3.4 Resistance to Cell Death

Many factors such as alcohol, viruses, toxic bile acids, fatty acids, drugs, and immune response cause cell death in liver. Cell death in nontransformed hepatocytes stimulates continuous cell turnover that provides a platform for cancer-initiating mutations and alterations in the composition of hepatic microenvironment acting as a tumor-promoting mechanism, mediated by increased compensatory regeneration, fibrogenesis, and inflammation. After malfunction of the death machinery by mutations, tumor cells often undergo a selection process that allows them to successfully evade cell death [114].

3.4.1 Hepatocyte Cell Death

Hepatocyte cell death during liver injury was classically viewed as either programmed (apoptotic), or accidental, uncontrolled (necrotic) cell death. In addition to these classical modes of cell death, several other forms of hepatic cell death have been described, including autophagic cell death and necroptosis. Apoptosis, a highly organized and genetically controlled process, is the most investigated and best defined form of programmed cell death. Apoptosis is initiated by either membrane receptors (extrinsic pathway) or intracellular stimuli (intrinsic pathway) and both pathways result in the activation of effector caspases 3 and 7, which execute the final apoptotic changes [115, 116]. Autophagy is a catabolic process controlled by the autophagy-related (Atg) proteins to mediate cellular homeostasis under basal and stressed conditions. To avoid confusion, the Nomenclature Committee on Cell Death (NCCD) reintroduced the term ‘autophagic cell death’ to describe cell death that is suppressed by inhibition of the autophagy pathway. There are molecular overlaps between regulation of autophagy and

apoptosis such as interaction of the BH3 domain of the Beclin 1 autophagy protein with the anti-apoptotic proteins, Bcl-2 and Bcl-XL. The autophagy may play a dual role in the development and promotion of HCC, as it may act as a promoter of tumorigenesis or as a tumor suppressor event [117]. Necroptosis is induced by the same death receptors that activate the extrinsic apoptotic pathway, namely TNF-R1, TNF-R2, and Fas. Upon interaction of receptor protein kinases 1 and 3 (RIP1 and RIP3), and in the absence of activated caspase 8, a cell death that morphologically resembles necrosis occurs [118]. Necrosis is an accidental form of cell death with fatal consequences. Cellular oxygen deprivation whereby the generation of reactive oxygen species (ROS) may lead to mitochondrial dysfunction and a drop in ATP levels below the threshold required to maintain cellular integrity, resulting in necrosis [115]. Morphologically, necrosis is characterized by cellular swelling, formation of membrane blebs lacking cellular organelles, and finally cell membrane rupture with the release of cellular contents [119].

3.4.2 Dysregulation of Apoptotic Cell Death in Hepatocellular Carcinoma

Apoptotic cell death is induced via membrane receptors and intracellular stress (Fig. 3.1). Both of apoptotic routes activate a variety of proteases, mainly the group of proteases called caspases (cysteinyl aspartate-specific proteases), and endonucleases, which finally degrade cellular components. Apoptotic events in hepatocytes are regulated by different stimuli that bind to death receptors in the cell membrane, such as Fas ligand (FasL), tumor necrosis factor-alpha (TNF-a) or TNF-related apoptosis-inducing ligand (TRAIL), which activate the extrinsic pathway. Furthermore, other factors, particularly the transforming growth factor-beta (TGF-β), do not bind to death receptors, but its intracellular signals may stimulate the apoptotic machinery through activation of the intrinsic pathway [116]. Binding of FasL, present in natural killer cells and cytotoxic T lymphocytes, or inflammatory cytokines, such as TNF-a, to their corresponding death receptors (Fas, TNF-R1) induces the recruitment of several adapter proteins and proenzymes (procaspase- 8 and 10) at the intracellular domain of the receptor to form the so-called death-inducing signaling complex (DISC). The signal generated at DISC by activated caspases leads to cell death, which, depending on the cell type, may or may not require the involvement of mitochondria for its execution [116]. TRAIL is a member of the TNF superfamily that can initiate apoptosis through the activation of their death receptors. It is regulated by two death receptors, TRAIL receptors 1 and 2 (TRAIL-R1 and TRAIL-R2), and two other decoy receptors. Because experimental evidence indicates that TRAIL induces apoptosis in liver cancer cells but not in healthy hepatocytes, TRAIL has been discussed as a promising alternative or additive therapeutic strategy [120]. However, the role of the TRAIL system in the pathogenesis of HCC is not clear yet.

The intrinsic pathway is triggered by different extra- or intracellular signals that induce mitochondrial dysfunction, resulting in altered membrane permeability and mitochondrial proteins being released into the cytosol, including proapoptogenic factors such as cytochrome c, SMAC/DIABLO (second mitochondria derived activator of caspases/direct IAP binding protein with low pI), apoptosis-inducing factor (AIF) or endonuclease G, among others. The release of cytochrome c from mitochondria promotes the formation of a complex between APAF-1 and caspase-9 in a caspase-activating structure known as the apoptosome [118] (Fig. 3.1). Several intracellular proteins are involved in the mitochondrial-mediated regulation of apoptosis, in particular, the Bcl-2 family of proteins, which includes at least 20 members of both pro- and anti-apoptotic effects, being one of the most important regulators of the intrinsic pathway (examples of anti-apoptotic proteins are Bcl-2 and Bcl-XL; examples of proapototic members are t-Bid, Bax and Bak). These proteins exert their effects upstream of the mitochondria integrating death and survival signals. The balance between pro- and anti-apoptogenic members and their interactions determine the intrinsic pathway initiation.

3.4.2.1 The Death Receptor Pathway

Hepatocellular carcinoma cells show resistance to apoptosis mediated by several death receptors. The majority of the HCCs show one or more alterations in the Fas pathway molecules, which inhibit Fas-mediated apoptosis. Loss of response to Fas in HCC cells and/or tissues is produced either by downregulation of *Fas* expression, concomitant with decreased expression of downstream molecules, such as FADD or FLICE or overexpression of cellular FLICE/caspase-8-inhibitory protein (cFLIP), or by upregulation or overactivation of molecules that counteract its pro-apoptotic effect, including nuclear factor-kappaB (NF-κB), Bcl-2 or Bcl-XL [121, 122]. Recently, it was shown that TRAIL expression is reduced significantly in about two-thirds of HCCs and this is correlated with tumor size and stage, recurrence after resection, and patient survival rate [123].

3.4.2.2 Apoptotic Regulatory Proteins

It is well known that there is an imbalance in the expression of pro- and anti-apoptotic members of the Bcl-2 family in HCC. Bcl-XL and Mcl-1 are overexpressed while pro-apoptotic members of the family, such as Bax or Bcl-XS are downregulated in HCC. Furthermore, some pro-apoptotic members of the BH-3-only family, such as Bid, show decreased expression

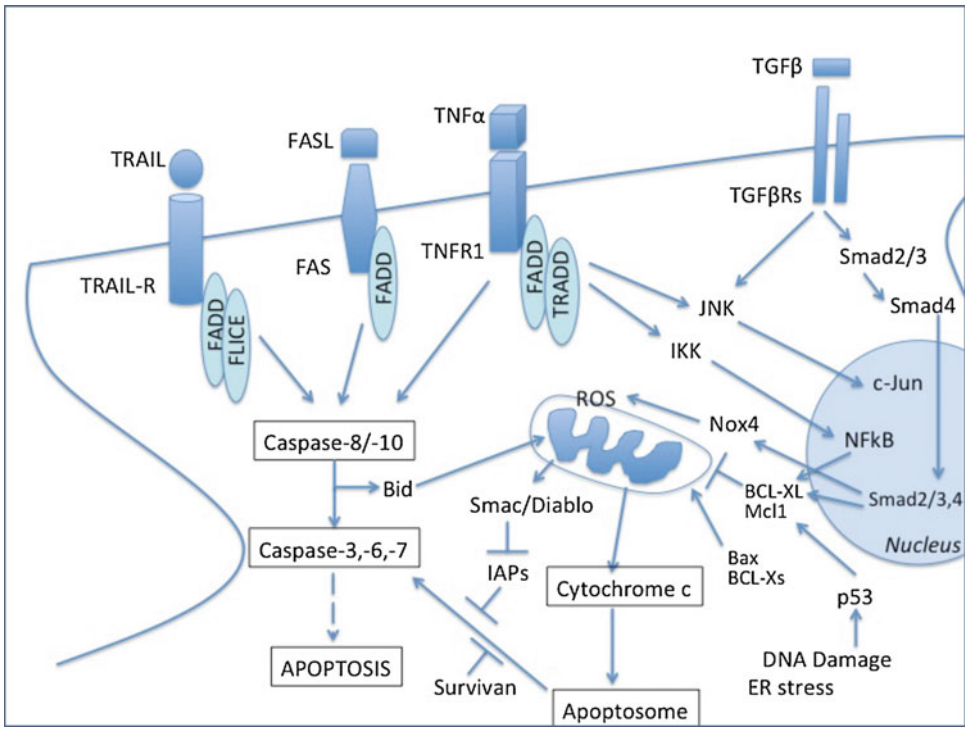

Fig. 3.1 Extracellular factors and intracellular signals controlling apoptotic cell death in hepatocytes

in HCC related to hepatitis B virus X protein or hepatitis C virus polyprotein (reviewed as Ref. [124]). Shi et al. 125 showed that nearly 90 % of clinical tumors from advanced HCC patients express high levels of X-linked inhibitor-of-apoptosis protein (XIAP), a well-known inhibitor of caspases and patients with XIAP-positive tumors have a significantly increased risk of relapse.

3.4.2.3 TGF-β Pathway and Apoptosis

TGF-β is normally produced by stellate cells and exerts its effects by limiting the growth of hepatocytes in response to injury by inhibiting DNA synthesis, blocking cell cycle progression and inducing apoptosis. The TGF-β family of cytokines plays also essential roles in cell migration and invasion, extracellular matrix remodeling and immune suppression, being involved in the maintenance of tissue homeostasis. Two types of catalytic receptors (TbRI and TbRII) have been described; they both contain and extracellular binding domain and intracellular serine/threonine kinase domain. TGF-β is first activated by proteolytic cleavage and then binds to TbR-II, which in turn recruits and trans-phosphorylates TbR-I. Transcription factors SMAD2 and SMAD3 are recruited to the activated receptor complex and activated by phosphorylation, being released from the complex and heterodimerizing with the mediator SMAD4, followed by translocation to the nucleus. Once in the nucleus, the activated SMADs and SMAD4 regulate transcription-forming complexes with other transcriptional coregulators [126] (Fig. 3.1).

The ability of TGF-β to induce or suppress programmed cell death varies greatly depending on the cell type [127]. In hepatocytes, it has been suggested that TGF-β induces the expression of the death-associated protein kinase (DAP-kinase) as an immediate SMAD-dependent early response (Jang et al. 2002). The adaptor protein DAXX has been also implicated as a mediator of TGF-β apoptotic signals because it physically associates with TbR-II, facilitating Jun amino-terminal kinase (JNK) activation (Perlman et al. 2001). The apoptosis induced by TGF-β also has been linked to an oxidative stress process, which is required for bcl-XL downregulation and mitochondria-dependent cell death [126]. This process might be associated with activation of TIEG (TGF-β-inducible early-response gene) and induction of a NAD(P)H oxidase-like gene, in particular NOX4 [126]. NOX family has emerged in the past years as an important source of ROS in liver pathologies. Interestingly, NOX proteins mediate some TGF-β actions in liver cells, such as regulation of hepatocyte growth and death, as well as activation of hepatic stellate cells to myofibroblasts, key executers of the fibrotic process. Furthermore, TGF-β impairs survival signaling events (such as PI3K/AKT pathway and c-IAPs), through activation of phosphatases and/or caspase-mediated proteolysis, respectively [128]. These various components of the TGF-β apoptotic programme ultimately couple the signal to the main components of the cell-death machinery (Fig. 3.1).

The disruption of the TGF-β signaling pathway occurs in HCC and might cause dysregulation of apoptosis.

Overexpression of SMAD3 reduces the susceptibility to develop HCC by sensitizing hepatocytes to apoptosis through downregulation of Bcl-2 [129]. HCC cells might also overexpress a specific set of microRNAs (miRNAs) that would allow the escape from TGF-β-induced apoptosis [130]. Furthermore, it has been indicated that TGF-β might play a dual role in controlling apoptosis in hepatocytes and hepatoma cells. On one hand, it induces cell death, but on the other it could activate anti-apoptotic signals, the epidermal growth factor receptor (EGFR) being required for this effect. Indeed, EGF is an important survival signal for TGF-β-induced apoptosis in hepatocytes. The enzyme phosphatidylinositol 3-kinase (PI3K) mediates the effect of EGF on TGF-β-induced death by acting upstream to the mitochondrial changes, probably counteracting TGF-β-induced oxidative stress [131]. The autocrine loop of EGFR activated by TGF-β in hepatoma cells would require a high activity of TACE/ADAM17, the metalloprotease responsible for shedding of the pro-tumor necrosis factor (proTNF-α) that it is also necessary for shedding of the EGF family of growth factors. Overexpression of TACE/ADAM17 might confer an advantage on HCC cells by impairing TGF-β-induced apoptosis through transactivation of the EGFR [132].

3.4.2.4 NF-κB *Pathway and Apoptosis*

NF-κB suppresses apoptosis by inducing the expression of a number of genes whose products inhibit apoptosis, including IAPs, cFLIP, TNF receptor associated factor 1 (TRAF1), and TRAF2. Two typical prosurvival NF-κB targets are Bcl-XL, an anti-apoptotic member of Bcl-2 family, and XIAP, a member of the caspases inhibitor, which are frequently overexpressed in HCC [133]. Interestingly, the NF-κB/Bcl-XL/XIAP axis potently counteracts the TGF-β-induced apoptosis and exerts a general cytoprotective role on preneoplastic hepatocytes. Recent results also linked NF-κB to the increase in the autocrine expression of EGF receptor ligands, such as TGF-a, in hepatocytes and hepatoma cells [134].

Different studies have implicated members of the NF-κB/Rel family in both HBV- and HCV-induced neoplastic development of the liver [133]. Several mechanisms have been proposed for activation of NF-κB by the hepatitis virus. Overall, inflammatory hepatitis might activate NF-κB by the concerted action of cytokines, such as TNF-a, chemokines or interleukins, and viral proteins, which likely will promote cell survival of precancerous hepatocytes [133, 134]. In summary, overactivation of the NF-κB pathway might generate resistance to apoptosis in HCC cells through different mechanisms.

3.5 Phenotypic Plasticity

Vertebrate embryo development requires an orchestrated cellular differentiation program associated with differential cell adhesion and tight regulation of dynamic cell contacts. E-cadherin regulated cell adhesion is a well-known process that affects both cell morphology and dynamic cellular interaction. Dynamic demands and plasticity require a tight regulation of the E-cadherin gene, *CDH1*. During key events, like epithelial-mesenchymal transition (EMT) and the reverse process of mesenchymal-epithelial transition (MET), the *CDH1* locus is shut down (EMT) and reactivated (MET). These processes are fundamental for normal development but are also exploited by tumor cells including HCC cells for dissemination and colonization during cancer progression.

3.5.1 Epithelial to Mesenchymal Transition

The process of EMT is utilized during embryogenesis at many key steps, such as neural crest delamination, heart valve formation, palatogenesis, and myogenesis (type I) [135]. Similarly, EMT becomes activated during fibrosis and wound healing (type II) and cancer (type III) [136]. Cytoskeletal rearrangements and modulation of the expression of many different genes, including cell adhesion molecules are common features of EMT. The key event of a bona fide EMT is the downregulation of E-cad that leads to loss of cell polarity, an adherent morphology and of the epithelial gene signature [137, 135]. In exchange, they acquire a mesenchymal unpolarized morphology combined with increased cell motility and a mesenchymal gene signature including the switching from cytokeratins to vimentin expression, increased fibronectin expression and activation of the PDGF/PDGFR autocrine loop allowing degradation of the ECM and thus promoting invasion [138]. Furthermore, activation of the expression of N-cadherin [137] among other cell surface proteins like CD44 [139] and integrin b6 [140] is thought to be important for the migratory phenotype.

Induction of EMT is integrated by many different developmental signaling pathways such as TGF-β, Wnt and Notch signaling [141]. In particular, the TGF-β signaling pathway has been described as the major inducer of EMT because of its direct activation of the core EMT regulators like Snail, Slug, Twist, Zeb1, Zeb2, and others [135, 142]. In agreement with a required fast downregulation of E-cadherin, all of these transcription factors are in fact repressors of E-cadherin expression and they all bind to several evolutionary conserved E-boxes present in the

proximal promoter [143–149]. Beside their role in the repression of E-cadherin expression, the EMT core regulators exert their effect on a multitude of levels ensuring a successful EMT. For example, Snail and Zeb family members have been shown to repress other adhesion molecules such as Claudins and ZO-1 [150, 151]. Similarly, Twist1 is able to orchestrate two events of EMT, it activates the expression of Snail2 ensuring the repression of Cdh1 [152], and also induces the expression of PDGFR which in turn activates Src, promoting invadopodia formation [138]. The complexity of coordinating the progress of EMT is a reflection of not only the complexity of the transcriptional networks orchestrating it [153], but also the complexity of the pathways inducing and governing EMT.

3.5.2 TGF-β Pathway and Cellular Plasticity

As mentioned above, TGF-β signaling pathway is considered as the major regulator of EMT. In healthy tissues, TGF-β plays a critical role in several cellular functions such as proliferation, differentiation, and apoptosis both in adult as well as in embryonic stages [154]. Beside the Smad proteins, TGF-β can also transduce its signals via other pathways such as PI3K and MAPK [155]. During tumorigenesis, cellular responses to TGF-β are different as the potent induction of EMT facilitates cell motility, invasion, and metastasis [156]. During EMT, the loss of E-cadherin expression results in the accumulation of b-catenin in the cytoplasm and eventually in the nucleus, leading to the activation of Wnt target genes [153] thus enhancing the Wnt signaling. This upregulation of Wnt pathway and the accumulation of nuclear b-catenin as a result of aberrant TGF-β signaling causes a proliferation of liver progenitor cells, which correlates with higher vascular invasion grades and increased recurrence of HCC [157, 158]. In HCC cells, inhibition of TGF-β signaling results in the upregulation of E-cadherin leading to the stabilization of an epithelial state with a lower migratory potential [159], suggesting the potential of developing novel therapeutic drugs targeting TGF-β [160].

3.5.3 b-catenin/Wnt Signaling Pathway and Plasticity

The canonical Wnt signaling pathway is an essential regulator of several cellular mechanisms such as proliferation and survival in adult cells and also plays an important role during embryonic development by regulating several developmental processes such as patterning, neurogenesis, and morphogenesis [161]. The b-catenin is considered as a key component of the canonical Wnt signaling pathway, in the absence of Wnt ligand, b-catenin is in a complex with E-cadherin at adherens junctions bridging adhesion molecules with the cytoskeleton, thus maintaining cellular polarity. Excess b-catenin is targeted for ubiquitination and degradation following phosphorylation by GSK3b, a member of the destruction complex that also includes Axin and APC. Upon binding of the ligand (Wnt) to the receptor (Frizzled) b-catenin escapes the destruction complex due to the destabilization of Axin, eventually inhibiting GSK3b activity, resulting in the accumulation of b-catenin, which then translocates to the nucleus where it activates the expression of Wnt target genes by modulating the activity of the TCF/LEF transcription factors [161].

Mutations and aberrant expression of several components of the Wnt signaling pathways have been reported in HCC patients leading to the activation of b-catenin, these include, but not limited to, b-catenin, Axin and APC mutations, as well as the overexpression of Frizzled and Wnt3 [162]. b-catenin mutanr mice do not develop liver cancer [163], but deregulated Wnt signaling may cooperate with other oncogenic pathways promoting HCC development [164]. The current scientific evidence favors the hypothesis that mutations of b-catenin are late events in HCC and thus may promote tumor progression rather than tumor initiation [162]. Many reports addressed the relationship between Wnt signaling and EMT, accumulation of nuclear b-catenin was shown to repress E-cadherin expression by either the transcriptional activation of the core EMT regulators Slug and Twist, or by the stabilization of Snail [165].

3.5.4 Notch Pathway and Plasticity

The notch pathway plays a critical role in several processes both in development and in adult cells. It is a key regulator of stem cell self-renewal, differentiation, and cell fate decision. In addition, Notch has been shown to be involved in proliferation, apoptosis, and EMT [166]. The activation of Notch signaling begins with the binding of the ligand (Delta or Jagged) to the Notch receptor. This binding causes the cleavage of the extracellular C terminal peptide, followed be cleavage of the intracellular domain (NICD), which then translocates to the nucleus where it activates the expression of Notch target genes [167].

Notch pathway has been shown to be involved in a variety of cancer types, such as breast, colorectal, pancreatic, and liver cancers [168]. In addition, Notch is well-known inducer of EMT, this is accomplished by the activation of Snail and Slug gene expression, resulting in the downregulation of E-cadherin and the initiation of EMT [169]. Recent evidence suggests a dual role of Notch signaling, depending on the cellular context it could promote oncogenic or tutor suppressive functions [170]. In HCC cells, the forced

expression of an active Notch results in growth inhibition due to cell cycle arrest, Notch 1 was shown to decrease the expression of several cell cycle modulators such as Cyclin A, Cycline D1, and Cyclin E. Moreover, increased Notch expression resulted in an increased expression of p21 and p53 [171].

3.5.5 Mesenchymal to Epithelial Transition

The reverse process of EMT, the mesenchymal to epithelial transition (MET) is a fundamental embryonic program as well [172, 173]. Here, mesenchymal cells acquire epithelial characteristics including loss of N-cadherin and activation of E-cadherin expression [135, 136, 142] and regaining of the polarized adherent morphology. The spatiotemporal reactivation of E-cadherin expression is critical for the progression of MET, forced silencing of Cdh1 expression during the onset of MET is sufficient to completely block the cells in a mesenchymal state [174]. This suggests the need for an immediate activation of the *Cdh1* locus during MET, a process which can only in part be explained by the downregulation of the core EMT regulators and their release from Cdh1 promoter [175, 176]. Recent evidence suggests that E-cadherin expression during MET is initiated by intronic enhancers and is a dynamic process involving several transcriptional regulators such as Grhl2 [177], Grhl3 and Hnf4a and p300 [174]. In addition to orchestrating morphogenetic events during embryogenesis, the process of MET is also utilized by disseminating tumor cells required for colonization and formation of metastasis at distant sites [135, 142].

3.6 Motility, Invasion, and Metastasis

Metastasis is a hallmark of cancer and the occurrence of intrahepatic and extrahepatic tumor cell metastasis is the primary factor causing mortality in patients with HCC [178]. Due to the dense hepatic vasculature and cirrhotic background, HCC shows intrahepatic multiple occurrence and intrahepatic metastasis. Tumors are multifocal within the liver 75 % of the time and extrahepatic metastasis of HCC occurs in about 30–50 % of patients in the late stages of the disease. The site of extrahepatic metastasis of HCC is most often the lungs and less often the lymph nodes and bones [179, 180].

Tumor metastasis is a multi-step program, and enhanced cell motility and invasion is a common feature of tumor metastasis. Tumor microenvironment is very important for tumor invasion and metastasis and is dramatically remodeled during tumor progression [181]. In this section, the key differences between the types of migration and invasion, the role of collective-amoeboid, mesenchymal-amoeboid, and amoeboid-mesenchymal transitions, as well as the significance of different tumor factors and stromal molecules in invasion and metastasis of HCC will be discussed.

3.6.1 Mechanisms of Cell Invasion

Both in vitro and in vivo studies have revealed a great diversity in the morphologies of invading cells and the way of cell migration. During cancer progression, a variety of tumor cells display morphological and phonotypical changes, and this plasticity enables tumors to adapt to microenvironmental conditions [182, 183]. Tumor cells invade the stroma either by moving as single cells or collectively and, conversions between collective to single-cell transition or vice versa has been described in tumor metastasis [183–185].

3.6.1.1 Single Cell Migration

This is characterized by the loss of cell–cell interactions and cells can migrate individually via two modes: mesenchymal-type and amoeboid-like [183, 185, 186]. Each pattern of cell migration that is observed in tumor invasion displays specific morphological features and the underlying molecular mechanisms of cell migration. The microenvironment plays a significant role in determining the morphology and migration mode of tumor cells, and depends on molecular changes in tumor cells [187, 181]. Like other solid tumors, HCC cells are capable of activating the mechanisms for changing their shape, creating conditions for moving, as well as remodeling surrounding tissues for migration [188].

Mesenchymal (Fibroblast-like) Cell Migration

Since malignant cells lose epithelial polarity and gain elongated spindle-shaped fibroblasts, the invasion of this type is called “fibroblast-like” migration [186, 183]. This motile phenotype of tumor cells occurs in a process called epithelial-mesenchymal transition or EMT [182, 189]. The mesenchymal mechanism of invasion is believed to be the consequence of EMT, when active dedifferentiation of a malignant epithelial tumor occurs, and multicellular groups start to divide into single tumor cells, gaining a mesenchymal phenotype [185, 186]. A spindle-shaped cell body and long protrusions are the characteristics of the mesenchymal phenotype [182].

Based on the suppression of the expression of the relevant genes using small interfering RNAs, the specific activity of GTPases Rac1 and Cdc42 was demonstrated to be the main characteristics of the mesenchymal type of invasion. Suppression of GTPase Rac1 through signaling activation of GTPase RhoA and its effector, ROCK kinase, leads to the

blockage of the mesenchymal migration of tumor cells. In HCC, the importance of Rho GTPases in the prediction of tumor metastasis was reported [190].

Recent literature indicated that the behavior of tumor cells during individual migration depends on the surrounding matrix stiffness [183, 185, 187]. Therefore, the key points of the fibroblast-like migration mechanism are the strong adhesion forces on both poles of the cell as well as between cells and extracellular matrix (ECM) components. The molecules, such as lysyl oxidase 2, that modify ECM stiffness is involved in the creation of a favorable microenvironment for tumor cells by activating fibroblasts and endothelial cells, and the activation of growth factors signaling in liver [191].

Amoeboid Cell Migration

The amoeboid-type cell migration of single tumor cells is one of the most efficient mechanisms of invasive growth. This type of migration has been described in circulating stem cells (CTCs), leukocytes, and certain types of tumor cells including HCC cells [182, 192]. In the case of amoeboid migration, malignant tumor cells have been demonstrated to have a round cell body phenotype and to differ the activity of their protrusions. Although amoeboid cells might have several variations, they are characterized by a round or elliptical shape, the development of "bleb-like" protrusions of the cell membrane, the fast deformability, the adaption of their shapes to existing structures of the surrounding extracellular matrix, and the penetration through them via narrow spaces in a compressed form [182, 183, 186]

Changes in the cell shape are generated by the reorganization of the actin cytoskeleton and controlled by the small GTPase RhoA and its effector, ROCK kinase [185]. It has been reported that activating the RhoA/Rho pathway increases amoeboid migration of HCC cells and the expression level of RhoA, which is directly correlated with the poor prognosis of HCC patients [190]. The activation of Hepatocyte Growth factor (HGF)/c-Met signaling induces the small GTPase system (Cdc42, Rac, RhoA, RhoC) that regulates the motility of tumor cells [185].

It has been reported that, in addition to the changes in cell shape, the shape of the nucleus and its orientation, and position relative to other internal organelles change during amoeboid migration of tumor cells [185]. Since tumor cells have to move through narrow spaces and pores, nuclei inside single migrating tumor cells move forward toward the leading edge. In contrast to the mesenchymal movement, amoeboid or a non-proteolytic model of migration prevails when the surrounding matrix is characterized by relatively low stiffness [185, 193].

The amoeboid mechanism is also characterized by a weak interaction between cells and the surrounding matrix, as well as a lack of, or weak, focal contacts. It was reported that integrins are not important in this type of invasive growth. Furthermore, the absence of proteolysis at the sites of cell–matrix interactions and the lack of expression of proteolytic enzymes and movement at the highest speed in cultures are main characteristics of amoeboid migration [185].

Due to the need to adapt to microenvironmental changes, a "shift" from one migration type to the other (*amoeboid-mesenchymal and mesenchymal-amoeboid transitions*) is possible. The expression and/or activation levels of proteases and protease inhibitors; the interaction levels of integrin receptors with surrounding ECM molecules; and the balance between the Rac and RhoA activity are important for these transitions [183, 185].

3.6.1.2 Collective Cell Migration

It can occur when groups of firmly interconnected tumor cells are migrating. Invading tumor cells in vivo typically preserve cell–cell contacts, leading to collective migration of cancer cells. It has been reported that the transition from individual to collective migration is an important step toward increasing the invasive and metastatic potential of malignant neoplasms [182, 184, 185].

Collective cell migration results from the establishment and maintenance of collective polarization, and is characterized by groups of cells that contain cell–cell adhesions and move as epithelial sheets or detached clusters. It is known as the slowest mode of cancer migration. Three hallmarks are reported to characterize collective cell migration: presence of intact cell–cell junctions during movement (*including adherence, tight and gap junctions as well as desmosomes*; multicellular coordination of cytoskeletal activity and polarity and the reorganization of ECM [185, 186].

Recent studies have shown that clusters of *circulating tumor cells (CTCs) and circulating tumor microemboli (CTM)* are present in the circulation of patients with metastatic cancers [192]. Circulating cells in the bloodstream or in the lymphatic system are representing a collective migration. It is also concluded that cells in the CTM arise from collectively migrating cells, rather than the aggregation of cancer cells [182, 184, 192, 194]. There are a few studies regarding the significance of CTCs or CTM in patients with HCC. These studies indicate that CTCs and/or CTM contribute to HCC recurrence, and may therefore serve as an important therapeutic target for the treatment, recurrence, and metastasis of HCC [195, 196].

In vivo data and pathology studies showed that cancer cells display epithelial or mesenchymal morphology, and that tumor cells within a single tumor can simultaneously move both collectively and individually. Migrating tumor cells (regardless of movement type) are more resistant to chemotherapy and radiotherapy than nonmoving cells [185].

Involvement of the same signaling pathways in cell migration and the development of tumor resistance to therapy were also reported [185, 197]. This might be related to the temporary loss of division ability in migrating cells. It may also be related to the increased activity of anti-apoptotic genes in moving tumor cells, which causes resistance to chemotherapeutic drugs aimed at the induction of programmed cell death. Recent data indicates strong association between collective migration and resistance to radiotherapy and chemotherapy in HCC cells [198].

During cancer metastasis, a variety of tumor cells show changes their plasticity by morphological and phenotypical conversions, including the *epithelial to mesenchymal transition (EMT)*, the *mesenchymal to epithelial transition (MET)*, the *collective to amoeboid transition (CAT)*, and the *mesenchymal to amoeboid transition (MAT)* [182, 185, 186].

As mentioned previously in this chapter, EMT plays an important role in the progression of HCC. During HCC progression, a variety of tumor cells show changes in their plasticity by morphological and phenotypical conversions [195, 189]. In principle, EMT is characterized by a loss of epithelial characteristics and gain of mesenchymal motility [185]. Furthermore, EMT is described to maintain stem cell properties, to prevent apoptosis and senescence, to suppress immune reactions, and to acquire chemoresistance [185, 199]. It is believed that EMT is activated by the factors in the tumor microenvironment including growth factors, hypoxia, and ECM components [188, 189].

EMT is a transient state that allows cancer cells to disseminate. However for metastasis that has to be reverted by MET at the metastatic site. MET, much less characterized at the molecular level, restores the specific epithelial identity. MET is also described as individual to collective transition and is important for colonization at secondary sites [186, 200].

The microenvironment in HCC is a complex mixture of tumor cells in ECM, combined with stromal cells and the proteins they secrete. Since HCC develops in chronically damaged tissue that contains fibrosis, inflammation, and angiogenesis, the role of the ECM in the initiation and progression in HCC is critical. Hepatic stellate cells and macrophages are important for the secretion of ECM components and growth factors that promote migration, invasion, neovascularization, and fibrosis [187, 188]. Liver fibrosis is characterized by an excess of ECM production and reduced ECM turnover. Deregulation of collagen crosslinking and ECM stiffness is important for integrin signaling [201]. Deregulation of ECM homeostasis and activation of integrin signaling lead to an excessive deposition of collagen types I and II and fibronectin in liver and activate several growth factors including TGF-β1, HGF, and EGF [202].

TGF-β is mostly expressed in stromal cells rather than malignant epithelial cells and is increased in HCC. At the microenvironment level, TGF-β is a key mediator of EMT, cell invasion, and angiogenesis in HCC. TGF-β also generates a favorable microenvironment for tumor growth [188]. While TGF-β1 treatment in HCC cells causes the downregulation of epithelial and hepatic markers (such as E-cadherin and albumin), it causes the upregulation of mesenchymal genes (such as vimentin and alpha-SMA) and an increase in motility and invasion [189].

HGF is another important mediator of EMT in HCC cells, as well as in fetal and adult hepatocytes [189]. It plays an important role in HCC tumor progression by promoting EMT, invasion, and cancer metastasis in cooperation with other pathways [203]. HGF/c-Met activation induces well-known EMT markers such as TWIST1, Snail, Slug, and ZEB1/2, which result in the disruption of strong cadherin junctions and the activation of cell migration and proteolysis of extracellular matrix components [189]. Many studies have shown that the overexpression of c-Met is correlated with a poor prognosis, including a risk of tumor recurrence and short [189]. We and others have reported that the overexpression of c-Met in HCC is linked to an unfavorable clinicopathological status, including a low degree of differentiation, vascular invasion, and metastasis [203, 204].

Several evidences strongly indicate that hypoxic microenvironment in liver promotes invasion and metastasis of HCC through inducing EMT [187, 189]. The possible associated molecular mechanism is that HIF-1 interacts with HREs in promoting Snail and upregulating the expression of Snail to indirectly affect expressions of E-cadherin, N-cadherin, and Vimentin [205]. In addition, HIF-1α acts as a transcription factor to upregulate the expression of matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9 [187, 188].

Hypoxia also induces VEGR expression and activates VEGF signaling which is a most important pro-angiogenic factor in the progression of HCC. The increased expression of VEGF correlates with HCC aggressiveness. Fibroblast growth factor (FGF) acts synergistically with VEGF to induce angiogenesis. Platelet-derived endothelial cell growth factor (PDGF) is involved in new vessel maturation. Other important mediators in tumor angiogenesis are integrins and cadherins, which mediate cell–matrix and cell–cell interactions, respectively, to establish contacts required for new vascular tube formations [187, 188].

Chemokines are important regulators of cell trafficking and endothelial cell migration in the tumor microenvironment. Injured hepatocytes, oval cells, biliary epithelial cells, sinusoidal endothelial cells, tumor-associated leucocytes, and HCC cells can release CXCL12. CXCL12 activates CXCR4-expressing cells, such as HCC cells, lymphocytes or

endothelial cells in an autocrine or paracrine manner. CXCL12/CXCR4 axis promotes angiogenesis and acting synergistically with VEGF. Tissues expressing high levels of CXCL12 attract malignant HCC cells expressing CXCR4. CXCR4 expressing HCC cells can migrate through CXCL12 gradient in target tissues, supporting a chemotactic role for this axis in the development of metastases [206].

Evidences of the role of CXCL12/CXCR4 on chemotaxis in HCC tumor progression have been provided by several reports showing high levels of CXCR4 in HCC tissues, but not in normal hepatic tissues [187, 207, 208]. The majority of studies show correlations between high CXCR4 expression and aggressive tumor behavior, metastasis development, and poor prognosis [207, 208]. Expression of the CXCR4 ligand, CXCL12, has been reported in tumor ascites fluid and detected in HCC lymph node metastases, whereas undetectable in HCC and normal hepatic tissues [207]. Furthermore, the importance of the *CXCL12-CXCR4 axis* in cell growth, migration, and invasion of HCC cells has been reported [206].

Tumor metastasis is a multifactorial process. Combination of tumor microenvironment, stromal network, and genetics determinants of tumor cells are important for tumor metastasis. Further studies are needed to understand the basic biology of tumor metastasis with the underlying cellular and molecular mechanisms. In particular, understanding the mechanisms of transition between collective, mesenchymal, and amoeboid mechanisms of cell invasion will help the development of novel strategies to combat metastasis.

3.7 Conclusion

Molecular mechanisms involved in hepatocellular carcinoma indicate that these tumors are characterized by a deregulation of mechanisms controlling the number and the phenotype of hepatocytes and/or their progenitors (Fig. 3.2). Deregulated cell cycle control, escape from senescence arrest, as well as resistance to cell death promote overall cell proliferation.

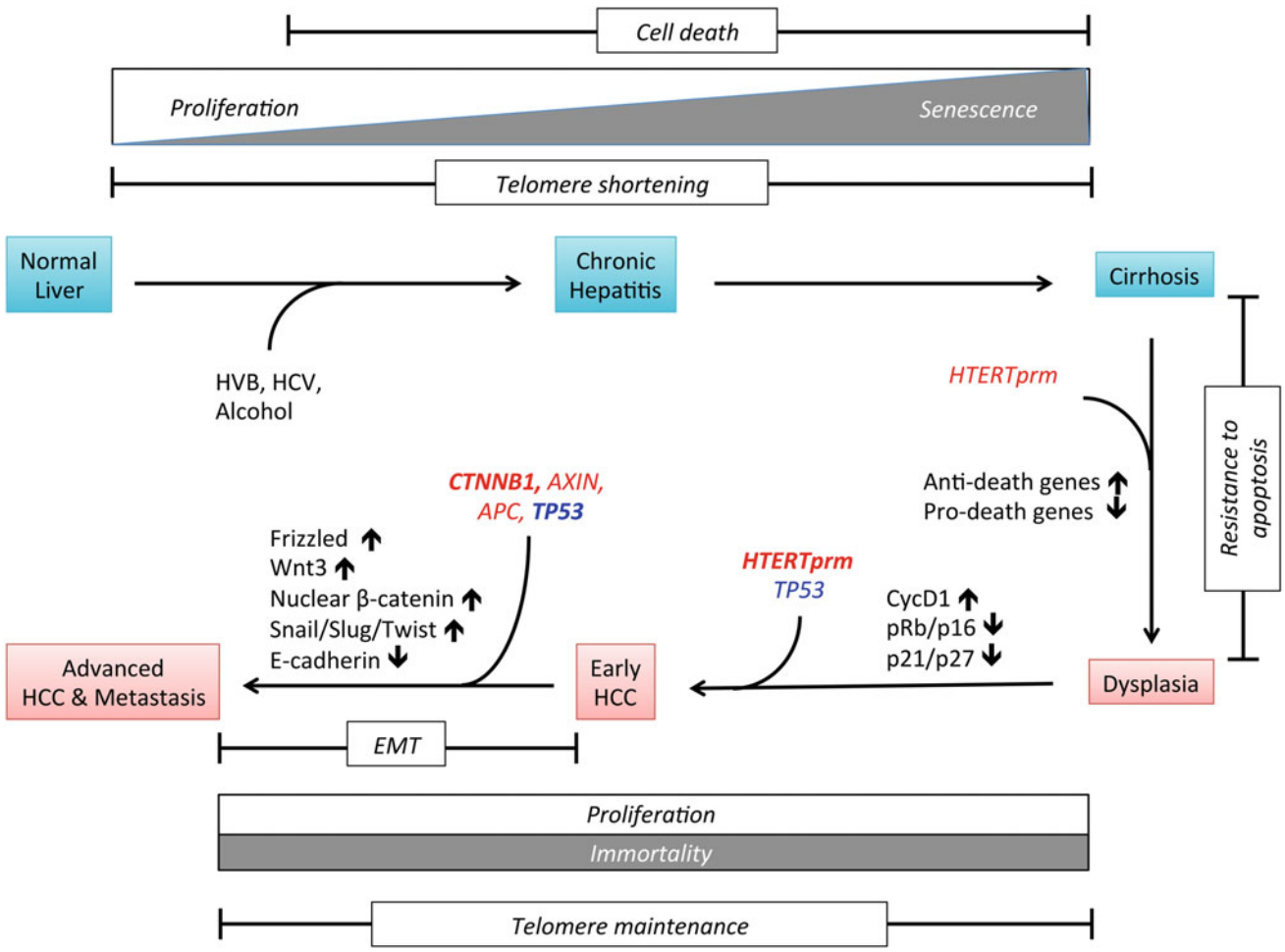

Fig. 3.2 Overview of molecular mechanism involved in hepatocellular carcinoma

Interestingly some of these changes can be explained by acquired genetic mutations in HCC such as p53 and p16 mutations, and TERT promoter mutations. However, many other aberrations are not presently linked to any known mutation. Future challenge will be the deciphering of non-mutational causes of cell growth anomalies in these tumors. Another major mechanism of malignancy is the loss of phenotypic stability observed in mature hepatocytes. Many HCC cells display a phenotypic plasticity allowing them to gain for example epithelial or mesenchymal morphologies depending on their needs. Another character is their increased motility that may help them to move from their resident space to other parts of the liver, even of the body. This faculty of changing morphology and behavior depending on environmental conditions may confer great survival and expansion advantage to HCC cells. Molecular changes associated to different phenotypic stages of HCC cells are well known. However, the mechanisms by which these tumor cells are able to modulate their phenotype are poorly known. Thus, another future priority in HCC research will probably be the mechanisms of phenotypic plasticity.

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Chemically-Induced Hepatocarcinogenesis

4

Yvonne P. Dragan

Contents

4.1 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 G0 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.

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 of neoplasms that arise

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at this site to man. The utility of defining common biomarkers for the conversion of benign to malignant transition will assist in developing appropriate inter-species extrapolation. The analysis of early lesions will permit assessment of the early changes that occur prior to the onset of clinically detectable disease to our understanding of HCC.

4.2 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 (750,000 cases/year) in terms of incidence [9]. In addition, it is the third most common cause of death (725,000 deaths/year) from cancer [10], with eighty percent of cases (and deaths) resulting from hepatitis B and/or C infection and occurring in developing countries. Surveillance Epidemiology and End Results [11], the National Cancer Institute's statistical unit, estimate that 35,000 new cases of liver and intrahepatic bile duct cancer were diagnosed and nearly 24,000 people will die from this disease in the US in 2015 [11]. Understanding the processes that contribute to the cancer development process is an important component 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 [12]. 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 2 million in the part of the US population sampled [13, 14]. Chronic infection with hepatitis C virus (HCV) is known to be a major risk factor for development of HCC. In general, HCC develops only after 2 or more decades of HCV infection and in those with advanced fibrosis [14, 15]. 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 US, 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 US [16]. 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 indicate 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.

4.3 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 and is the result of distinct etiologies and pathogenesis. 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 [17]. 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 their 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.

4.3.1 Genotoxic Carcinogens

Chemically induced carcinogenesis has been examined experimentally for nearly 100 years [18, 19]. Initial studies provided the compounds typically in the diet for extended periods of time. For example, the studies of Sasaki and Yoshida [20] demonstrated that chemicals could cause hepatic neoplasms in animals. Provision of o-aminoazotoluene in the diet led to liver neoplasms in rats. Similarly, Kinoshita [21] 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 [22]. 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 [23]. Similarly, certain azo dyes, while carcinogenic to the liver, do not have this activity in the skin [24]. The agent 2-acetylaminofluorene but not its related regioisomer, 4-acetylaminofluorene, is carcinogenic in the rodent liver [25]. However, dialkyl nitrosamines and several analogs are cytotoxic to the liver and are carcinogenic in rodents and many other mammals [26]. 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 Aspergillis flavus is acutely cytotoxic. This agent is also carcinogenic in all species examined, although the mouse is relatively resistant to its carcinogenic action [27]. A variety of other agents in food can also be carcinogenic to the liver including certain mycotoxins [28] in addition to aflatoxin (fumonisin in rodents) and pyrrolizidine [29] alkaloids (found in comfrey and riddelline). In addition, a dearth of antioxidants and a lack of lipotropes [30, 31] can lead to cancer development in the rodent.

4.3.1.1 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 [32]. Many of 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 when they are coupled with cell proliferation mutations can result [33]. 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 [24]. The biological consequences of these actions differ. Early studies by Miller and 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 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 is more carcinogenic than the parent AAF [24]. 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 result in more reactive agents with facile leaving groups. This is the case for some esters of *N*-hydroxy AAF [24]. 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 of agents that are positive in rodent bioassays have yielded reactive groups that yield structural alerts for carcinogenic risk [39, 40].

4.3.1.2 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 [42]. 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 [43]. For example, aflatoxin B1 can be metabolized to 8,9 epoxide of AFB1, which then binds to N7 guanosine leading to mutations [44]. Mutation of G to T can occur at multiple sites, most notably at 249Ser of P53 [45]. Methylation, ethylation, and other alkylations can occur with each of the bases as well as the sugar and phosphate backbone [46, 47]. 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 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 [48]. Methylation or ethylation of DNA can lead to base mispairing [46, 49]. 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 [50, 51].

The consequence of bulky adduct presence is to block DNA synthesis resulting in noncoding [47]. However, the DNA synthetic machinery can bypass such lesions placing in its stead the most abundant nucleotide, generally an adenine [52]. Since bulky adducts typically occur at guanines, this is a useful endogenous 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 metabolized to the sulfate ester that is highly reactive; binding to the N7 of guanine as well as the N3 of guanine [24]. 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, 2AF can induce point mutations, while 2AAF can lead to frameshift mutations [53]. Biological consequence of the presence of DNA adducts is a function of their persistence in the DNA [54] 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 [33]. 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 [55], and immune system [56] 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 [57–59]. For example, oncogenes such as Ha-ras can be activated by a single point mutation [60]. Activation of Ha-ras is an important mechanism of HCC induction and development in the mouse [42, 61], but not in rats or humans [19]. In the liver, activation and mutation of β-catenin (and possibly axin) is an important aspect of some types of liver cancer [62, 63]. 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 been described with non-random mutations. 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 [63].

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 [64]. Repair processes for oxidative damage are pervasive in most cell types nonetheless oxidized bases can persist [65]. Although all of the DNA bases can be oxidized, the most common are 8-hydroxy deoxyguanosine [66] and 5-hydroxymethylthymine [67]. These oxidative bases likely arise through endogenous processes [68] and they are readily repaired. The most prevalent endogenous modification of DNA is methylation of deoxycytidine [69, 70]. 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 [71]. Diets deficient in lipotropes can result in marked steatosis followed in time by HCC formation in rodents [31]. Methyl deficient diets can result in DNA hypomethylation. Global hypomethylation results in re-expression of genes in general, while hypermethylation results in their silencing [72]. Perturbation of nucleosomes, of 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. Both endogenous and exogenous derived DNA alterations can result in cancer initiation [64].

4.3.1.3 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 [33]. Thus, the rate of cell proliferation and DNA synthesis can impact DNA damage [73]. 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 [47] 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 [74, 75]. 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 [76] and a nonhomologous end joining process is used that is error prone [77]. Mismatch repair can occur when bases are mispaired or when it appears that they are mispaired due to the presence of a DNA modification [78]. 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.

4.3.2 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 [19, 79]. Certain agents are cytotoxic at either high doses or with chronic administration [80]. These agents such as chloroform do not pose a risk when exposure occurs below the threshold for cytotoxicity [81]. For example, chronic high dose ethanol consumption results in high levels of acetaldehyde generation [82]. 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 [83]. 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 over consumption 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 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, 19, 79]. 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 for the selction of initiated cells [27].

4.3.2.1 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 [92, 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 [99, 100]. 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 was observed [100]. 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 that are responsible for apoptosis [101, 102]. 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 [102, 103]. Phenobarbital can promote those initiated cells with a low level of TGFβR, while increasing ligand expression in surrounding hepatocytes [102–104]. 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, 104].

Previous work has demonstrated that Phenobarbital-like compounds cause the increase in gene expression of a number of genes including CYP2B1/2 [105] and is transcriptionally regulated [106]. The tumor promoting action of this type of agent is correlated with the induction of CYP2B1 [107]; 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 [108]. Agents that act to alter the metabolism of testosterone derivatives, specifically androstenedione, can alter endogenous activation of the CAR receptor [109]. There are two forms of CAR and Phenobarbital can displace the ligand from CARβ [109]. Agents such as phenobarbital activate the CAR receptor to perturb gene expression [110–113]. 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 [113, 114]. 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 [112]. Nonetheless, CAR knock-out mice are resistant to Phenobarbital induced hepatic tumor promotion [114]. Interestingly, chronic Phenobarbital administration results in DNA hypomethylation that is CAR-dependent [115]. 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 [116], lack CAR, and are not promoted by phenobarbital [117]. These tumors lack CAR, but express β-catenin and are promoted by phenobarbital [117, 118].

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 CAR is a nuclear receptor that regulates the expression of drug metabolizing enzymes [110–113]. 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 have 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 [119]. The mode of action of phenobarbital for hepatic tumor promotion has been reviewed [120].

4.3.2.2 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 [121–126]. Estrogenic agents can increase cell proliferation in the rat liver and can induce focal proliferation with mitosuppression in the surrounding hepatocytes [127, 128]. 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 [129, 130]. The increase in TGFβ leads to CKI induction that may lead more directly to the mitoinhibition [131]. Similarly, EE exposure induces TGFβ1 expression. Hepatocytes with decreased levels of TGFβR are at a selective growth advantage compared to cells without this characteristic [102]. Hepatocytes that survive TGFβ exposure have decreased HNF4α activity, but increased fos, jun, myc, and ras levels [132]. Oncogene expression can confer tumor characteristics that TGFβ responsiveness can limit [133]; 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 [134–136].

Sustained estrogen receptor activation is known to increase the incidence of liver neoplasms in animals and humans [137–140]. 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 were observed in women taking early formulations of estrogens for oral contraceptive purposes [90, 137]. Estrogenic agents are effective tumor promoting agents in the rat liver and their action to initiate cells through catechol estrogen formation [138] or induction of aneuploidy [139] needs to be assessed at physiological concentrations. For example, certain estrogenic agents can cause a burst of increased proliferation in the rodent liver [140]. This transient increase in cell proliferation is associated with stimulation of the estrogen receptor [124, 128]. There is a mitosuppression in the normal appearing hepatocytes, while the focal, putatively, preneoplastic hepatocytes have a sustained increase in proliferation [128, 129, 141]. 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 [90]. Estrogenic agents are known liver tumor promoting agents in the rat [122, 123, 135] and in the human [142]. There is an apparent threshold for promoting action [142–144]. 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 [145]. In addition, tamoxifen can inhibit the development of mestranol promoted lesions indicating a divergent mechanism of action [124, 135]. 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 [146], tubulin [147], and other interactions with "antiestrogenic binding sites" [148]. In addition, antiestrogens inhibit protein kinase C and calmodulin activity [149]. In addition, antiestrogens alter the production of several peptide growth factors including TGFα [150], TGFβ [151], and IGF1 [152], and affect some calcium dependent processes [153]. Estrogenic and antiestrogenic agents additionally alter cholesterol metabolism [148]. Tamoxifen appears to promote the diploid hepatocyte population [154], similar to ethinyl estradiol [155]. The triphenylethylene antiestrogens have differential effects on the hepatic proliferative rate in the rat [156, 157]. 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 [158] to the potent rat liver tumor-promoting agent, ethinyl estradiol [150]. Mestranol use in oral contraceptives was associated with an increased incidence of hepatic adenomas and a few HCCs in young women [90, 159–161]. 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 [162], but it can cause cholestasis [163] and clearly enhances liver growth

[162]. Chronic administration of ethinyl estradiol results in mitosuppression of liver cells with selection of resistant hepatocytes for outgrowth [127, 128] and this in combination with its ability to increase cell proliferation [124, 164]; is believed responsible for its tumor promoting properties [121–124, 127, 128, 144, 165, 166]. Tumor promotion by ethinyl estradiol is effected through the estrogen receptor, since it can be inhibited by tamoxifen [135, 136]. At low doses and for short durations of administration, ethinyl estradiol can increase hepatic hypertrophy and a transient increase in cell proliferation [124, 164], while with chronic administration a mito-inhibition is observed [124, 127].

4.3.2.3 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 [167]. The PPAR alpha receptor is a ligand activated nuclear transcription factor that is responsible for the regulation of lipid catabolism [168]. 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 [169–171]. Peroxisome proliferators include structurally diverse chemicals that can activate the PPARα receptor including industrial chemicals, plasticizers, herbicdes, and some lipid lowering drugs [171–173]. Agonists of PPARα induce peroxisome proliferation [173, 174], hepatomegly [173, 175], cell proliferation [173, 176, 177], and liver neoplasms in rodents [171, 177, 178]. 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 [179]. 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 [180]. Sustained PPARα receptor activation is required for induction of liver tumors, since PPARα knock-out mice do not develop hepatic neoplasms even after a one year exposure to a PPARα agonists [181]. Similarly, peroxisome proliferation and gene expression regulated by PPARα are not altered by exposure to PPARα agonists in the knock-out mice [181]. The lack of carcinogenic action in the human relative to the rodent has been explored with human PPARα receptor knock-in mice [182]. Although the precise mechanism of the hepatocarcinogenesis of PPARα agonists in rodents is not fully understood, it appears dependent upon PPARα receptor activation [183–185]. Thus, PPARα agonists are non-genotoxic carcinogens that function through receptor activation [186] and appear to be carcinogenic in the rodent, but not in primates.

4.3.2.4 AhR Agonists

The aryl hydrocarbon receptor (AhR) is structurally distinct from the nuclear receptors, and contains a bHLH-PAS domain [187–189]. 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 [190–192]. In addition, the transactivation domain part of AhR is highly divergent with only a 60 % identity between rat and human [192]. 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 [187]. Dioxin lacks any genotoxic activity, yet increases the incidence of hepatic neoplasms in rats [193]. 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 [194]. 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 [195]. Genetic differences between mouse strains have been used to demonstrate that TCDD-mediated liver tumor promotion is AhR dependent [196]. 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 [197]. Knock-out animals have been generated [198–200]. The gene expression patterns [201] and toxicity [202] 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 [203] suggesting that the C3H background would be exquisitely

sensitive to TCDD induced tumor promotion [119]. When IL1-like knock out mice are generated on an AhR knock-out background, hepatic tumor induction by TCDD is decreased [203] similar to the dual receptor dependence on the IL1R and AhR receptor for TCDD-induced hepatotoxicity.

Initiation-promotion studies in the rat [204, 205] 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 [206, 207]. However, an increased cell turnover in focal lesions was noted relative to the surrounding liver [208, 209]. The initiated cell population is resistant to apoptosis [209, 210]. Interestingly, the AhR null hepatocytes both secrete TGFβ ligands and are quite sensitive to the apoptosis induced by TGFβ [210], 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 [201] and the acute toxicities associated with AhR activation are diminished [202]. For example CAR is increased by AhR activation [211], while growth hormone receptor and janus kinase 2 are decreased [212]. 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 [213, 214]. In part, the human AhR receptor is less sensitive to activation by AhR ligands [192] and in part, the exposure level in humans has been below that required to cause sustained tumor promotion [214]. Other agents in the class including certain of the 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 carinogenicity 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 [215], 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 [216]. The risks at high dose exposure differ from the risks posed by ambient exposures, sine multiple modes of action occur at the higher exposures.

4.3.2.5 Ethionine

Ethionine is 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 [30]. 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 [31, 217]. 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 [217]. This agent is not used in the human.

4.4 Pathogenesis of HCC

The pathogenesis of human HCC has been examined extensively [6–8, 218]. 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 multiples 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.

4.4.1 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 [219, 220]. 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 [221]. 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 2 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.

4.4.2 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 [222], the Solt-Farber model [223], and transgenic [224] rat models can be used to analysis 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 in which to examine the process of liver cancer development and to ascertain which compounds can influence cancer development in the liver. Studies by Bannasch [225] indicate that two pathways that evolve toward HCC in the rat are thyroidmimetic and insulinmimetic (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 [16]. 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 [225]. 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 [225, 226].

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 [223]. Preneoplastic lesions have a higher level of expression of genes that are anti-apoptotic (p53, NK-kB and Bcl-2 pathways) and pro-proliferation [226]. Proliferation gene changes are also common in liver tumors, while apoptosis was decreased [227, 228]. Early nodules demonstrate a decrease in both growth hormone receptor and growth hormone binding proteins [229]. Specifically, IGF2 is expressed during liver cancer development, while IGF1 is decreased during liver cancer development [230]. These more fetal-like gene expression patterns are observed during early tumor development [231]. The increased expression of TGFα and HGF and their respective receptors, EGFR and met, observed in early nodules is lost with neoplastic progression [232]. Gene expression analysis demonstrates many genes in common between neoplastic nodules and HCC with only a few genes uniquely observed in HCC [226, 232].

4.4.2.1 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 two days of carcinogen exposure [233–238]. Under many conditions, GST expression has been suggested to represent a population of initiated hepatocytes in the rat liver [235, 236, 238]. This is true for several types of genotoxic carcinogens including diethylnitrosamine [233, 238], an alkylating agent, aflatoxin B1 [233] that results in the formation of bulky DNA adducts, and choline deficient diet that result in depletion of methyl pools [237]. Single GSTP expressing hepatocytes are found in a dose-dependent manner following carcinogen administration [233]. 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 [233]. Expression of genes at the single cell level has been inadequately characterized, but GSTP and GGT are increased in certain hepatocytes following carcinogen administration.

4.4.2.2 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 [233]. The hepatocytes within AHF during promotion are primarily diploid [239, 240] and additionally lack demonstrable karyotypic changes [240]. 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 [234]. 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.

4.4.2.3 Progression

The stage of progression encompasses the spectrum of changes that occur in the conversion of preneoplastic cells into malignant neoplasia [32]. 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 [32, 222, 241, 242]. Interestingly, gene expression differences between resistance and sensitivity of rat strains to liver cancer progression have been described [243].

4.4.3 Mouse Models

Certain mouse strains are more susceptible to spontaneous [244] and chemically induced [245] hepatic tumors than other strains. An upregulation of c-jun may mark single altered cells in the mouse liver [246] 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 6Phosphatase. 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 [247]. 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 [248]. In a study performed by Drinkwater et al. [249], 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 [250]. Strain differences in sensitivity to liver cancer development were described by Andervont [244] 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 [247, 251]. Studies with other mouse strains and other carcinogens have also been performed [252].

The National Toxicology Program assesses cancer risk in the B6C3 F1 mouse that carries the dominant susceptibility allele for liver cancer development. The most common experimental cancer assessment tool is the neonatal mouse model [253] as first described by Vessilinovitch [254]. Numerous models of human liver diseases exist. Many of these are developed as a complicated toxin or carcinogen

regimen [18]. In addition, genetically modified mice have been made against signaling pathway members believed important in liver cancer development [224]. These rarely are a complete recapitulation of the human disease, but are nonetheless useful for modeling one component of the disease [224]. The challenge is to couple etiologic agents, with pathway perturbations and disease models to unravel components of the pathogenesis of human primary liver cancer [18, 224, 255]. 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.

4.5 Etiology in the Human

Patients at risk for HCC include those with chronic hepatitis B virus (HBV) and/or HCV infection [14, 256], certain metabolic liver diseases, such as hereditary hemochromatosis [257], Wilson's disease, α-anti-trypsin deficiency, and porphyria cutanea tarda [7, 8]. Individuals with cirrhosis are at risk of HCC [7, 258]. Heavy alcohol consumption is also a common major risk factor for developing HCC [7, 8, 83, 85, 258]. Other predisposing factors include gender (males are times more likely to develop HCC than females), smoking, and diabetes [258]. 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 [259]. Thus, primary liver cancer is a product of environmental exposures with genetic consequences. In the US, 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 US are not associated with HBV or HCV) [8]. The incidence of HCC is increasing in the US primarily due to an increase in HCV infection [8]. It has also been proposed that the rising incidence of obesity, type 2 diabetes, and non-alcoholic liver disease contributes to this increased incidence of HCC [120].

4.5.1 Cirrhosis

Individuals with cirrhosis, regardless of its etiology are at risk for HCC [7, 258]. Fibrosis of the liver can result as a response to liver injury or as a component of selected genetic diseases [260, 261]. Cirrhosis is the endstage 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 [262]. 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 provides other contributing factors [7, 8]. There is an increased risk of primary liver cancer in individuals with hepatitis C associated cirrhosis and diabetes mellitus [263]. In some conditions, cirrhosis can progress to HCC.

4.5.2 Non-alcoholic Steatohepatitis (NASH)

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of elevated serum enzymes indicative of liver injury and may be due to many etiologies [264–269]. 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 [264, 265, 267]. Essentially all morbidly obese individuals have NAFLD and approximately 25–50 % exhibit steatohepatitis. For NASH patients (prevalence of 1–5 % in the general population) approximately 20 % will progress to cirrhosis, with a small percentage of these progressing to HCC. Approximately 10 % of individuals with NASH will die of liver related diseases [265, 266]. NASH is common in type two diabetes and has a prevalence of 60 % [265–267, 269, 270]. 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 NASH. In some individuals, steatohepatitis can progress to cirrhosis and in a limited number of cases can progress to primary liver cancer [270]. 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 [271, 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].

4.5.3 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 have100 fold risk of developing HBV [14] 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 % [14]. 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 [279] in concert with the greater prevalence of chronic hepatitis and cirrhosis in men [279]. A prospective study of liver cancer development among men in Taiwan has indicated a relationship between serum testosterone levels and risk for HCC [279, 280]. Men, whose testosterone levels was 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. When other risk factors, including HBsAg carrier status, anti-HCV positivity, and alcohol consumption, were take into account, the relative risk for men with high testosterone levels was 4 [14, 278]. 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, 5 286]. 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 [14, 278].

4.5.4 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 [27, 281]. The risk is heightened in those with HBV [282]. 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 [27]. 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 [283]. 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 fumonosins [284]. Other fungal [285, 286] and microbial products [287] 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 [288]. These include the pyrrolizidine alkaloids are found in comfrey, and riddeline [289]. The use of Senecio, Crotalaria, Heliotropium, and Synphytum 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 [290–294].

4.5.5 Alcohol and Tobacco

Alcohol abuse has been associated with HCC development that occurs in a background of hepatitis and cirrhosis [258, 295]. 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] and a higher rate of HCC are observed in heavier 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 at least 2 fold [87].

4.5.6 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 were 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 HCC development [259, 279]. 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 [259].

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 [142] 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 [142, 316]. 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 [317]. Case-control studies in the United States, Britain, and Italy demonstrated a 5-fold increased risk for HCC among women with more than 5 years use of oral contraceptives relative to women with exposures of shorter duration [315, 317–319]. In contrast, estrogen replacement therapy does not increase the risk for HCCs [315]. Thus, excess exposure to hormonally active agents can increase the risk of HCC.

4.5.7 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 α1 antitrypsin. Individuals with cirrhosis and genetic hemachromatosis have a markedly increased rate and shortened time until HCC development that is exacerbated by viral infection and alcohol abuse [273, 279]. Other metabolic diseases can increase the risk of HCC but to 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.

4.5.7.1 Metal Overload Disorders

Iron overload [257, 320, 321] has been associated with hepatic fibrosis, cirrhosis, and HCC. Hereditary disturbances in iron uptake [322–324] and metabolism results 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 [320, 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, 258]. 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 knockout mouse, periportal iron deposition in conjunction and elevated transferrin saturation [327]. Interestingly, HFE and B2M are in a complex with transferrin receptor HFE results in an increase in intestinal iron absorption. HFE mutation carriers cannot facilitate iron update by transferrin receptor resulting in an upregulation of the iron responsive gene dimetal transporter 1 that enhancing iron uptake [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 [321].

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 [257, 320]. The incidence of HCC in HH is increased over 100X relative to a comparative control population [257, 320]. Outcomes in heterozygotes for HFE seem similar to wildtype, except for those 1–2 % individuals who are compound heterozygotes with C263Y/H63D [331, 332]. 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 [332]. 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 [331–333]. The increased risk from HFE alleles is found in alcoholic cirrhosis and some cases of HCV viral hepatitis, but not HBV viral hepatitis patients [331, 333]. Animal models of liver disease in combination with iron overload also demonstrate an increase in disease progression [334]. 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 [334]. A wide range of hepatic tumor phenotypes is observed in human HFE [335]. Interestingly, a high incidence of p53 mutations has been observed in one series of HCC from HFE patients [336]. 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 [337].

Wilson's disease or inherited copper-overload disease can result in cirrhosis, hepatitis, and HCC. Wilson's disease is found in 1:30000 with a carrier rate of 1:250 [338]. Ceruloplasmin 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 [339]. 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 [338]. 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 ceruloplasmin. 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 [340, 341]. 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 M1356 V and G712D have defects in copper transport [342] and a knock out mouse (ATP7B) has also been generated [343]. 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.

4.5.7.2 Alpha-1 Anti-trypsin

Alpha-1 Anti-trypsin (AAT) is a prevalent protease inhibitor (Pi) found in the plasma [344]. The most prevalent mutation is a Glu342Lys caused by a G to A transition called the Z mutation [345, 346]. Adult males that are homozygous for

the Z mutation (PiZZ) may have an increased risk of cirrhosis and HCC [345–347]. Alpha 1 antitrypsin results in an increased risk of HCC in the absence of cirrhosis in homozygotes [347]. Carriers (PiZ) are also believed to be at an increased risk for HCC [348] especially in combination with other risk factors [349, 350]. 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 [345, 346].

4.5.7.3 Hereditary Tyrosinemia

Tyrosinemia is an autosomal recessive disorder that can lead to HCC. This inborn error of metabolism results [351] from inactivation of fumaryl acetoacetate 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 [352, 353]. A mouse model has been developed in which FAH is knocked out [354]. This mutant recapitulates the pathogenesis of human hereditary tyrosinemia type 1 and can be protected by nitisinone [355]. Intervention with nitisinone does not reverse gene expression changes associated with tyrosinemia [356]. 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 [357].

4.5.7.4 Citrullinemia

The inborn errors of disease associated with the urea cycle [358, 359]; namely, mutation of arginosuccinate results in acute liver toxicity [360]. 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 hyerammonia 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 [360]. A knock out mouse has been generated that has high citrilline blood levels and a severe hyperammonemic phenotype [361, 362]. The aspartate-glutamate carrier (AGC), SLC25A13, gene mutations result in citrin deficiency [363] and may develop hepatic steatosis and steatohepatitis [364]. These type 2 citrullinemia patients have an increased level of pancreas derived trypsin inhibitor and are associated with pancreatistis [363]. A decrease in this mitochondrial ACG, citrin, results in hepatic apoptosis through a caspase pathway in which the bax to bcl2 ratio is inverted [357]. A knock-out model has been described, but does not recapitulate all of the pathologies associated with adult onset type 2 citrullinemia [363]. The citrin/mitochondrial glycerol-3-phosphate dehydrogenase double knock-out mutant is a better model for type 2 citrullinemia [365]. Urea cycle disruption and perturbations of nitrogen removal can have adverse effects on the liver as exemplified by citrullinemia.

4.5.8 Genomic Landscape of HCC

The genomic landscape of cancer has evolved as a concept in cancer to account for the many genetic changes observed in neoplasms [366–368]. It has been suggested that primary hepatocarcinoma has an average of 6 mutations per megabase of DNA [369]. This high number may in part due to the late stage of life in which the cancer is detected as well as the late stage of its lifecycle when it is detected. The genetic changes observed in cancer especially liver cancer are considered to have an environmental and lifestyle component reflected in the genetic and epigenetic changes observed [370]. The recent ability to deeply sequence whole exomes or entire sequences as compared with single genes has emphasized this point. While many genetic signatures have been detected in neoplasms [366–368], six have been demonstrated in liver cancer using COSMIC [369; http://cancer.sanger.ac.uk/cosmic]. Specifically, the genetic landscape of hepatocellular adenocarcinoma has been associated with the etiology of the disease, while the stage of disease has been more correlated with the expression and pathway alterations although these two factors and sets of changes are interdependent. One of the primary genetic signatures present in HCC (COSMIC signature 1B) is that of C > T that has been associated with aging. This may in fact reflect oxidative stress that is prevalent in cirrhosis and in viral and alcohol induced liver cancer and which can be found in aflatoxin excess. In this situation, a helix-distorting adenine adducts at GpCpN on the transcribed strand are contributory. Similarly, diseases such as NAFLD/NASH and hemochromatosis also have ongoing

oxidative stress and damage that would contribute to this type of genetic signature and to HCC development. A second signature (COSMIC signature 5) has a similar, albeit less prominent pattern of C > T changes that in this case are associated with dinucleotide mutation and strand synthesis bias. In the third signature (COSMIC signature 6), interstitial deletions at nucleotide repeats are common. This microsatellite instability is associated with mismatched repair deficiency resulting in high C > T, lower C > A, and even lower levels of T > C. The fourth identified gene mutation pattern for HCC (this is COSMO signature 4) is associated with the transcribed strand and has not been associated with a single predominant mutation, but rather may be associated with the infidelity of the polymerase and of transcription-coupled repair. In the fifth signature associated with HCC (COSMIC signature 16), a high level of T > C is observed and has been associated with transcription-coupled repair. In addition, a high level of T to C transversions is associated with the presence of G adducts as are frequently observed following polyaromatic hydrocarbon exposure as observed, although not exclusively, with tobacco smoke and exposure to other combustible products. A final predominant signature associated with HCC has a high level of T > G and a medium amount of T > C changes (COSMIC signature 17). The genetic landscape of a cancer reflects the cumulative environmental exposure, the impact of underlying liver disease, the etiology of the neoplasm, and its pathogenesis. This has been examined extensively in liver cancer for p53 and ras loci, but has now been extended across the genome. This whole genome examination has been instrumental in deciphering the complexity and heterogeneity of HCC. Genome wide analysis is now possible with the combined development of deep sequencing and big data based bioinformatics approaches. Besides mutations, insertions, deletions and amplifications, copy number variants and other factors that alter gene expression. In addition, mechanisms that impact gene dosage are important in liver cancer development and progression.

With respect to gene expression, a number of kinases and potentially phosphatases are of importance in altered gene expression in the liver and with liver cancer development [370, 371]. Specifically, Met, EGFR, and IGFR families have been implicated in liver cancer development and progression. Other receptors including VEGF2, PDGF, and FGF have roles in HCC pathogenesis. In addition, downstream signaling pathways (MAPK and AKT) and transcription factors (ras, mTOR, and have been implicated in HCC development and progression. One of the most important signaling pathways associated with HCC is the WNT pathway [370, 371]. An inflammatory mechanism is associated with some HCC and may be associated with estrogen-dependent regulation of IL6, NFkB and other mechanisms including those that signal through JAK/STS and TGFb. Recent, studies of mutations in HCC have confirmed the incidence of mutations in p53 and beta-catenin. Furthermore, the many mutations have been mapped against pathways and network to reveal the importance of proliferation, apoptosis, tumor microenvironment, neural signaling, metabolic pathways, and circadian pathways [371]. These pathways include cell cycle, p53 signaling, Wnt, MAPK, PI3 K/AKT and apoptosis, but also calcium signaling and Hippo pathways based on TGAC analysis. While these pathways are associated in general with HCC, their association with etiology, pathogenesis, and prognosis requires additional analysis. Additionally, chromatin- remodeling genes are altered in HCC. These include ARIAD1a/d, ARID2, MLL, MLL3, TERT among others [372]. The advent of deep sequencing as applied to the whole genome or all exons in conjunction with improved bioinformatics tools and well characterized sample banks of well defined pathology samples and their accompanying metadata have enabled important insights into the genomic landscape of liver cancer as demonstrated with the TGAC and COSMIC databases [373, 374].

4.5.9 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 background of a variety of genetic alterations and disease backgrounds. 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 disregulate in different genetic and disease backgrounds to lead to HCC development and progression.

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Molecular Profiling of Human Hepatocellular Carcinoma

5

Anuradha Budhu and Xin Wei Wang

Contents

5.1 Hepatocellular Carcinoma: Clinical Concerns

The wide heterogeneity of HCC and the complexity of its diagnostic and prognostic assessment (dependent upon tumor grade/residual liver function contributed by various etiological factors) 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 [1–6]. Although individuals at high risk for HCC development are routinely screened by ultrasonography and serum alpha-fetoprotein (AFP), most patients are diagnosed at advanced disease stages. AFP evaluation however, can be nonspecific, vary significantly between ethnic groups and is only observed in a HCC subgroup with small tumors [7]. 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 [8–10]. 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 renders HCC treatment especially difficult [11]. 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 postsurgical survival is complicated by a predom-

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inant occurrence of tumor recurrence/metastasis [12–14]. Methods to improve survival include percutaneous ethanol injection, radiofrequency ablation, and transarterial chemoembolization (TACE) [15, 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.

5.2 Molecular Profiling: Technologies and Platforms

The gene expression profile of a particular cell type or tissue has been analyzed in earlier years 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), metabolites (metabolomics), and genetic aberrations (array CGH/methylation) [17–19]. In addition, sequencing on the DNA and RNA level has also increased our capacity to identify the mutation landscape of HCC [20–22]. Although previous methodologies to study HCC have advanced the field, molecular profiling of clinical samples from HCC patients and HCC-related cell lines have enriched the breadth of HCC knowledge and have allowed researchers to begin to tackle some of the key disease-related concepts that still remain.

5.2.1 Molecular 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 multiple layers of signaling information including the genome, epigenome, transcriptome, proteome, and metabolome. A brief overview of widely used array platforms is provided below.

5.2.1.1 Genomic Profiling (aCGH, Methylation, Sequencing)

Array Comparative Genomic Hybridization

An important method of identifying driver genes involved in HCC is to detect genomic regions that undergo frequent alterations or are modified. Several types of alterations are present in the liver including changes in gene copy number, mutations, and chromosomal rearrangements. Array Comparative Genomic Hybridization (aCGH) using the BAC-based (Bacterial Artificial Chromosome) and oligonucleotide-based CGH enables high-resolution multi-loci mapping of small genomic regions with copy number changes, such as amplification or deletion [23, 24]. BAC aCGH is limited by costly, time-consuming, low-yield clone production and noisy data due to nonspecific hybridization of repetitive sequences. Oligonucleotide aCGH allows for flexibility in probe design, greater genomic coverage, and higher resolution ($\sim$50 kB). 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 requires a high concentration of high-quality BAC DNA for good array performance [25, 26]. Recently, genome-wide approaches, such as the single nucleotide polymorphism (SNP) 6.0 arrays, have allowed for global analyses of copy number alterations in HCC. Using these methods, numerous amplified and deleted genes have been observed in HCC.

Methylation

A few CGH array studies have been followed by bisulfate DNA sequencing or methylation-specific PCR to identify HCC-related epigenetic changes [27–29]. Since HCC develops against a background of chronic liver damage, the extent of genetic and epigenetic alterations is essential for our understanding of this cancer. In particular, methylation at CpG sites in gene promoters can affect the transcription of important genes in cancer. In fact, several hypermethylation events have been observed in tumor suppressor genes, suggesting a role for carcinogenesis promotion via this disruption of normal transcriptional events and induction of chromosomal instability. Indeed, certain methylation events have been associated with HCC patient survival and

recurrence and targeting of the epigenetic machinery has been the basis of some trials for HCC therapy [30, 31]. Methylation events can occur in several sites, including gene promoters, gene bodies, repetitive sequences, and intergenic regions, however the functional importance of specific alterations currently remains unclear. The induction of methylation events is also largely unknown, although some studies have shown that hepatitis viral infections, as well as nonalcoholic fatty liver disease can induce changes in methylation [32, 33]. Comprehensive methylation profiles of HCC are now readily studied by array-based platforms such as the Human Methylation 450 Bead Array and next-generation sequencing technology [34]. Our understanding of the HCC epigenetic code may allow for the development of novel diagnostic and therapeutic approaches for HCC.

Sequencing

High-resolution assessment of the liver cancer genome is now possible through advances in next-generation sequencing technologies [35]. An in-depth exploration of the liver genome has recently been employed through whole exome sequencing. This method is based on the capture or enrichment of DNA fragments containing the exonic region followed by massively parallel sequencing to determine somatic mutations [36–38]. Using this technology, several somatic alterations in the protein-coding region have been identified in HCC. To further identify somatic drivers in HCC, efforts have also been made to sequence the entire liver genome. This is referred to as whole genome sequencing whereby structural rearrangements, substitutions in noncoding regions, and viral integration sites can be explored. These methods however, look at rather short lengths of DNA sequences and thus, the identification of large genomic alterations is still rather limited. Although several key molecules have been indentified or validated by these methods, there seem to be a large number of passenger mutations present, which makes the identification of key driving genes in HCC a more complex problem.

RNA sequencing meanwhile, has added to our capacity for transcriptome profiling by allowing us to explore rearrangements in transcripts, noncoding RNAs, and splicing events. This highly sensitive method provides a more accurate tool for measuring expression across the transcriptome. Transcript abundance is quantifiable using this method, along with the identification of both known and novel features in the coding and noncoding transcriptome. Overall quality of starting samples, sequencing libraries, sequencing coverage, as well as time and cost parameters can have a significant impact on the sensitivity of detection and data quality in these types of experiments. These comprehensive genomic analyses however, are enabling researchers to examine the liver cancer genome at a much higher resolution with potentially impactful findings that could advance clinical management of this disease.

5.2.1.2 Transcriptomic Profiling (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 [39]. Multiplexed target profiling of hundreds of transcripts is also readily available through newer applications such as Nanostring [40]. In addition, the regulation of mRNAs can be analyzed using noncoding arrays (e.g., microRNA, pre-microRNA, snoRNA), which globally interrogate the expression of small endogenous (21–35 nt) RNA species. Platforms that detect mature and precursor forms of >2000 miRNAs are now commercially available [41–43].

5.2.1.3 Proteomic Profiling (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 posttranslational modifications, and correlate proteins with disease advancement or with certain treatments/environments [44]. Tissue microarrays (TMA) allow tissue-based profiling using small cylinders of formalin-fixed tissues arrayed in a single paraffin block [45]. 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 may help to resolve this problem [46]. Comprehensive proteomic characterization has been performed for certain cancer types, such as colon and rectal, however there is currently a lack of such studies for HCC [47].

5.2.1.4 Metabolomic Profiling

Cancer metabolite profiling (metabolomics) is a promising new approach to understand the biological mechanisms underlying cancer development and progression. Metabolomics provides a global view of metabolites, the

biochemical end products of cellular processes, enabling the characterization of cancer through metabolic changes, whose regulation are tightly linked with a certain pathological state [48]. In fact, metabolites are the best molecular indicators of cell status, since metabolic fluxes can change in a matter of seconds versus the comparatively slower turnover of mRNA and proteins [49, 50]. Thus, metabolic alterations are an extremely sensitive measure of cellular phenotype. Although genomics-based studies have been performed to extensively profile human tumors [51–56], relatively little is known about the global metabolite alterations that characterize cancer and how all of these events are intertwined as a network leading to aggressive disease and poor outcome. A systematic assessment of the pathways in which these genes and biochemical molecules contribute may lead to a more precise set of alterations that may serve as key biomarkers or drug targets for clinical interrogation in cancer patients suffering dismal prognosis.

5.2.2 Computational Analysis

Methodologies for analysis of large-scale omics data can be either unsupervised or supervised [57, 58]. Unsupervised methods attempt to characterize the components of a dataset without a priori input or knowledge of a training set. Internal structure or relationships in datasets are found by feature determination which groups genes/molecules with interesting properties (principal component analysis), cluster determination which groups genes or samples with similar patterns of gene/molecule expression or abundance (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 molecular features that fit a predetermined pattern [59]. This technique finds genes/molecules with expression or abundance levels that are significantly different between groups of samples (e.g., cancer classification) and can be used to find genes/molecules that accurately predict a characteristic of that sample (e.g., survival or metastasis). The significance found by supervised methods has been evaluated using parametric, nonparametric, and analysis of variance procedures which involve permutations, random partitioning of the studied dataset, 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 or abundance, including absolute or ratio of expression or abundance 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. Corrective statistics are also used when identifying genes/molecules of interest, to account for multiple testing in large datasets, including adjusted *p*-value, false discovery cutoffs, and Bonferroni corrections [60]. Due to the high complexity and sheer magnitude of current datasets, such as those ensuing from sequencing studies, new techniques and methods are constantly being explored, updated, and created to adequately analyze data. Many of these methods rely on algorithms and codes, most based on the R programming language, and in-house or stand-alone software associated with new technologies. A gold standard has been proposed for analysis of array studies which involves the use of a training dataset to initially identify a signature, a test dataset to assess its predictive/classification capacity, and an independent set for validation studies [61–63]. Importantly, biomarkers and signatures of interest need to not only be tested in retrospective cohorts, but also in prospective studies and in context of therapeutic strategies for HCC.

5.3 Tumor Signatures

Array studies have provided vast amounts of information concerning the genes, proteins, metabolites, and genomic changes that occur in HCC-related disease. These investigations have revealed changes that occur across the spectrum of cirrhosis, HCC tumors, the HCC microenvironment, HCC subtypes, epigenetic alterations, and progressive phenotypes (metastasis/recurrence). A general overview of these studies along with a synopsis of emerging perspectives gleaned from these analyses is provided in this section.

5.3.1 Tumor-Based Diagnostic HCC Signatures

5.3.1.1 Tumor Biomarkers (Tumor Vs. Nontumor)

Array studies have enhanced our understanding of how the HCC process alters the regulatory network of genes, proteins, metabolites, and epigenetic effects, in a way that differs from the respective normal tissue or disease-free samples. For example, cDNA analysis of HCC versus normal samples have found 38 differentially expressed genes while HBV-related cell lines revealed signatures (356 genes) composed of upregulated ribosomal-related genes [64, 65]. TIPUH1, a regulator of transcription and RNA processing of growth control genes has also been shown to be upregulated in HCC by cDNA array [66]. It has also been 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 [55]. A cDNA array of non-HBV/HCV-infected HCC versus normal tissues revealed 61 differentially expressed genes [67]. 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 [68–70]. Integrin and Akt/NFKB signaling were also upregulated in HCC along with a serum biomarker (CSTB) using cDNA arrays [71, 72]. Similar studies have shown that activators of neutrophils, anti-apoptotic genes, interferon response genes and proteins related to cell differentiation or development are differentially expressed in HCV-HCC [73]. 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 [74]. Several of these pathways, along with growth factor alterations were found in cDNA arrays comparing HBV or HCV-positive tumor versus nontumor tissue [75]. A clear distinction was found between HBV and HCV samples, where HBV affected genes 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 versus nontumor 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 [76].

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 nontumor tissue [77]. Other HCC-related protein classifiers include those involved in heat shock response, glycolysis, fatty acid transport and trafficking, amino acid metabolism, cell cycle regulation and cell stress, and metabolism related enzymes [78–80]. 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 [81]. Quantitative proteomics revealed that the SET complex is associated with HCC [82], while complement C3a was suggested as a HCC biomarker in HCV-HCC [83]. Serum monocyte chemoattractant protein-1 and prolactin have also been identified as potential tumor markers in HCC [84]. A TMA study of HCC versus nontumor found HCC-specific expression of the transcription repressor Zinc fingers and homeoboxes 2 (ZHX2) protein expression which correlated with differentiation stage [85].

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 [86, 87]. In addition, a study of 120 HCC samples found LOH at 6q and 9p in small, well-differentiated tumors [88]. A comparison of tumor versus nontumor 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 [87]. In a study of 20 HCC cases, oncogenes were amplified in 1q, 8p, and 11q regions while loss occurred at 13q and 4q [89]. A study of HCV-HCC revealed that increases of DNA copy number were frequent at 10p while decreases were frequent at 10q [86]. 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 HBV-HCC, gains on 1q, 6p, 8q, 9p were observed while losses in 1p, 16q, and 19p occurred in most patients [90]. 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 [91]. 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) [92, 93].

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 [94, 95]. miRNA array studies have also demonstrated that miR-21 can contribute to HCC growth and spread by modulating PTEN [96]. 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 [97–99]. In another study comparing HCC samples and adjacent nontumor, 8 miRNAs were shown to be significantly altered, 5 of which were downregulated in HCC and could predict HCC with 97 % accuracy [100]. More recently, microRNAs present in the circulation have also been identified as potential biomarkers for HCC [101, 102].

DNA methylation-based prognosis and epidrivers for HCC have also been studied. Villaneuva et al. identified a signature of 36 DNA methylation markers that predicts HCC patient survival and harbor mRNA signatures of tumors with progenitor cell features [103]. Deng et al. applied methylated DNA immunoprecipitation to identify 15 genes preferentially methylated in HCV-HCC [104]. Using a 27 K

Infinium array, thousands of differentially methylated genes in HCC were found, several of which could be assayed in plasma [34]. Tumor from nontumor specimens could be readily indentified in a methylation study of HCC using a 450 K array. Methylation events in p53, CTNNB1, GSTP1, MGMT, RASSF1A and in promoter CpG islands of CDKN2A have also been identified in HCC [105–108].

Array-based comparisons have also been made between early neoplastic stages (fibrosis/cirrhosis) and HCC. A study of 59 preneoplastic chronic liver diseases (CLDs) including hepatitis, autoimmune hepatitis, primary biliary cirrhosis found genes associated with high or low risk of HCC development [109]. This 273-gene signature was validated in three independent cohorts and included 12 secretory genes in the top geneset. 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 [110]. In an OLIGO array-based study of fibrosis, carbohydrate metabolism genes were elevated in HCC patients when compared to cases with F3–4 fibrosis [111]. 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 [112]. An RT-based study of cirrhosis versus HCV-HCC showed that eight genes were significantly altered (GPC3, TERT, Survivin, XLKD1, and CDH1) [113]. MiRNA platforms have also demonstrated that 35 miRNAs including let7 and miR-181 family members differ between HCC and cirrhosis [114]. Circulating microRNAs have also been shown as important modulators in early stage HCC [115]. aCGH of 63 HCCs found etiology-dependent copy number gains, including 8q24 and MYC overexpression in viral and alcohol-related HCCs [116]. 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 [117, 118]. In other studies, and CD5L and Annexin A2 were found as discriminative candidates in HCC [119, 120].

5.3.1.2 Tumor Biomarkers (Epigenetic Signatures)

HCC development is thought to be a multistep process, not only involving accumulation of genetic changes, but also epigenetic changes, such as methylation, which can reversibly alter regulatory genes. Several 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 [121]. In another cDNA array and bisulfite PCR study, insulin-like growth factor binding protein was found to be hypermethylated and downregulated in HCC [122]. 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 [123]. In addition, Pang et al. found a loss of an unmethylated 6q allele in HCC encoding a putative tumor suppressor gene [124]. 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 [125]. A promoter methylation study of 30 HCC tumors showed that they exhibit specific DNA methylation signatures associated with major risk factors and tumor progression stage, with potential clinical applications in HCC diagnosis [126].

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. Across platforms however, marker sets are quite different from one another, despite a similarity in comparison groups which could be due to platform makeup, sample heterogeneity, etiological differences, or ethnicity among samples. In addition, many of these studies lack validation and are only drawn from relatively small datasets 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.

5.3.2 Tumor-Based Prognostic Signatures

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 [127]. In HCC however, such stepwise and specific progression-related genetic changes have not been illustrated.

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 [128]. 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 [129, 130]. 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 [131]. 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 [132]. Another cDNA study of HCC found 217 genes associated with differentiation status and metastasis, including ANXA2 [133]. Another cDNA-based study found that HCC with high expression of ubiquitin-cojugating 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 [134, 135]. 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 [136, 137]. cDNA arrays have also been used to show that intrahepatic metastatic lesions are indistinguishable from their primary HCC while primary metastasis-free HCC was distinct from primary HCC with metastasis [53]. 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. It has also been investigated whether certain miRNAs are associated with HCC metastasis [138]. 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 ($p < 0.01$) [139]. 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 [85]. 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) as well as Rac and VEGF, key angiogenic factors in cancer progression, were correlated with HCC metastasis by TMAs [140, 141]. 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/beta-catenin pathway [142]. Another study revealed that loss of 17p13.3 and 8q11 were independent prognostic indicators of poor HCC patient survival [143]. LOH has also been observed at 16q and 17q in HCC and occurred more frequently in metastatic lesions [144]. The authors suggest that upregulation of PFTK1, in particular, may confer a motile phenotype in malignant hepatocytes that correlates with metastasis. Proteomics has also been applied to understand HCC progression. Tan et al. recently used comparative proteomics to identify proteins to differentiate patients who relapse from those who do not [145]. Proteomics has also been used to identify Talin-1 upregulation to be associated with HCC prognosis [146].

Tumor recurrence complicates resection in a large percentage of cases due either to 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 [147]. A cDNA-based study of 18 HCCs found a 14-gene signature that differed between vascular invasion status and could predict postresection recurrence [148]. 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 [149]. Meanwhile, a 12-gene OLIGO array-based signature has also been shown to predict recurrence within 1 year postsurgery with 93 % accuracy [150]. 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 was an independent risk factor associated with recurrence in a multivariate analysis [151]. 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 [152]. 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) [153, 154]. cDNA arrays have also been used to identify a 46-gene signature associated with extrahepatic recurrence [155]. The 20-miRNA metastasis signature identified was also significantly associated with recurrence in early stage HCC [138].

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 nonoverlapping, 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.

5.4 Microenvironment Signatures

Studies have suggested that while tumor cells affect metastatic capacity, the organ microenvironment can also contribute to this phenotype [156–158]. To determine the role of the hepatic microenvironment in HCC metastasis, the cDNA profiles of noncancerous surrounding hepatic tissues (n = 115) from HCC patients with venous metastases, termed a metastasis-inclined microenvironment (MIM) sample to those without detectable metastases, termed a metastasis-averse microenvironment (MAM) sample were compared [54]. A unique change in the gene expression profiles associated with a metastatic phenotype was identified which was refined to 17 immune-related genes. This signature was inherently different from a signature found in HCC tumor tissues and was validated in an independent cohort (n = 95). The nontumor 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 to 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. Gene expression profiling of nontumor specimens from HCC patients was also used to identify a molecular signature from formalin-fixed paraffin-embedded tissues. This poor prognosis signature was related to impaired liver function and inflammation, particularly interleukin-6. In addition, Hoshida et al. demonstrated that profiles of the surrounding nontumoral liver tissue were highly correlated with survival among Japanese, US, and European patients with HCC [159, 160]. These findings help to solidify the role of the field effect, whereby environmental exposures may play a role in tumor development and progression.

It has also been demonstrated that the expression levels of certain small RNAs, termed microRNAs, are altered in HCC metastasis. In a follow-up study, this 20-microRNA signature was validated and the role of a particular microRNA, let-7g in HCC progression, was determined [161]. It was confirmed that the level of let-7g was significantly lower in metastatic compared to nonmetastatic HCC and was predictive of poor survival. Functional studies indicated that let-7g could significantly inhibit cell migration and cell growth through targeting of soluble collagens. These results suggest that let-7g may suppress HCC metastasis through targeting collagen and that let-7g could be used as a tool to predict poor survival.

Given the predominant underlying fibrotic and cirrhotic conditions of the liver in those individuals prone to HCC and its recurrence, alterations of components of the inflammatory milieu have been suggested as factors which propel the formation and advancement of HCC. In particular, the activity of hepatic stellate cells (HSC), key features of fibrosis and cirrhosis, have been suggested as contributors to the HCC-prone microenvironment. A HSC-specific gene expression signature among tissue specimens of 319 HCC patients was recently identified and validated that is significantly and independently associated with HCC recurrence and survival [162]. Further computational analyses and immunohistochemical validation in a cohort of 143 HCC patients showed that the majority of alterations in patients with poor prognosis defined by HSC status were associated with peritumoral, rather than tumoral tissues. Furthermore, coculture studies demonstrate that HSCs preferentially affect monocyte populations, particularly CD14+ cells, within the microenvironment, that are related to a Th2-cytokine promoting shift in their inflammatory state. The interactions between HSCs and monocytes induce protumorigenic and progressive features of HCC cells by enhancing cell proliferation, migration, and tumor sphere formation. In sum, these results show that HSCs play a significant role in promoting HCC progression via interaction with and alteration of monocyte activities within the liver microenvironment.

Another hepatic stellate cell signature was recently identified in hepatitis C patients and was validated retrospectively in HCC patients to identify those with poor prognosis [163]. Thus, disrupting the interactions and signaling events between the inflammatory milieu and components of the microenvironment may be useful therapeutic strategies for preventing HCC tumor relapse. In addition, Tao et al. analyzed hepatocytes isolated from HBV-HCC cases on a 27 K array and identified hypermethylated genes. Overall, these studies highlight the significant role of the field cancerization effect to initiate and drive cancer progression [164]. More recently, other factors, such as the diet and the microbiome, are being studied to determine their roles in influencing the liver microenvironment [165].

5.5 Tumor Heterogeneity and Subclassification

Tumor heterogeneity may result from different cells of origin, range in patient ethnicity, etiology, underlying disease, and diversity of genomic and epigenomic changes which drive tumor development. Molecular differences between tumors from different patients, intertumor heterogeneity, and between different areas of an individual tumor, intratumor heterogeneity, have been recognized, possibly emanating from the presence of cancer stem cells or selection by clonal evolution. Cancer genomic heterogeneity thereby results in varying degrees of clinical presentation and tumor biology, which impedes treatment options and poses a significant challenge to cancer management [166]. An emerging challenge in HCC clinical management is intratumor heterogeneity, whereby distinct cell populations within a given tumor may result in poor response or resistance to therapy [167]. Some initial attempts have been made to characterize the extent of intratumor heterogeneity in HCC. In a recent study of 120 tumor areas from 23 HCC, intratumor heterogeneity measured by morphology, imunohistochemistry, and/or gene mutation status was found in the majority of specimens [168]. A comprehensive omics approach geared toward this feature of tumor biology is necessary for improving HCC clinical management. Findings of this type indicate that single tumor biopsies and the data collected from such specimens may not provide the entire portrait of alterations occurring in a given tumor. This nonuniformity of molecular changes currently represents a significant challenge in the development of targeted therapy for HCC.

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 either related to IFN-associated inflammation or apoptosis while another cDNA study composed of 19 HCC cell lines, found two subtypes that were correlated with AFP expression [169, 170]. 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 [171]. A separate cDNA study of HCCs from 10 patients found several genes related to histological subtype [172]. In an OLIGO study of well-differentiated HCC versus hepatocellular ademonas, 63 genes were found to be differentially expressed, demonstrating molecular differences despite similarities in morphology [173]. Another OLIGO study identified 31 genes that differed between early and advanced HCV-HCCs [174]. 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 [175, 176]. TMA has also been applied to identify tumor subgroups. Recently, Tan et al. applied comparative proteomics to HCC tumor tissues and identified a three-protein panel (HSP70, ASS1, and UGP2) that could stratify HCC patients into two groups [145]. A miRNA-based classification of three subclasses of HCC has also recently been proposed [177]. Among the proliferation class, miR-517a is an oncogenic miRNA that promotes tumor progression. Thus, there is a rationale for developing therapies that target miR-517a for patients with HCC.

We recently hypothesized that AFP^+ and AFP^- HCC tumors differ biologically. Using global microRNA profiling, we found that miR-29 family members were significantly downregulated in AFP^+ tumors with a significant inverse correlation between miR-29 and DNMT3A gene expression [178]. We also showed that AFP^+ and AFP^- HCC tumors have distinct global DNA methylation patterns, with an increased DNA methylation in AFP^+ HCC. AFP expression induces protumorigenic features along with miR-29a inhibition and DNMT3A induction. AFP also inhibited transcription of the miR-29a/b-1 locus via c-MYC binding to the miR-29a/b-1 transcript. Further, AFP expression promotes tumor growth of AFP^- HCC cells in nude mice. Thus, tumor biology differs considerably between AFP^+ HCC and AFP^- HCC and that AFP is a functional antagonist of miR-29, which may contribute to global epigenetic alterations and poor prognosis in HCC.

Recent attempts have been made to utilize profiling data to molecularly classify HCC in order to identify common homogenous subgroups of this disease which may respond more preferably to certain types of treatment. Studies indicate that aberrant activation of signaling pathways involved in cellular proliferation (e.g., epidermal growth factor and RAS/mitogen-activated protein kinase pathways), survival (e.g., Akt/mechanistic target of rapamycin pathway),

differentiation (e.g., Wnt and Hedgehog pathways), and angiogenesis (e.g., vascular endothelial growth factor and platelet-derived growth factor) are present in particular groups of HCC tumors [179, 180]. These cancer genes are thus ideal targets for biotherapies, underscoring the importance of tumor biology to medicine.

5.6 Stem Cell-Based Signatures

The heterogeneic nature of HCC and variability of its prognosis suggests 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 [52]. 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. cDNA arrays were used to identify a HCC subtype with features of hepatic stem cells that expresses AFP and a cell surface hepatic stem cell marker, EpCAM [56, 181]. EpCAM-positive cells from this subtype have self-renewal and differentiation traits and can initiate highly invasive HCC in NOD/SCID mice [182]. 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.

It was 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 [183]. A global microRNA microarray approach was used to explore whether certain microRNAs were associated with HCC stem cells. It was found that the conserved microRNA-181 family members were upregulated in HCC stem cells. Inhibition of microRNA-181 led to a reduction in number and tumor initiating activity of HCC stem cells while addition of microRNA-181 led to an enrichment of this cell type. In further studies, microRNA-181 could directly target transcriptional regulators of differentiation in the liver and an inhibitor of Wnt-beta-catenin signaling. In addition, Wnt/beta-catenin signaling transcriptionally activates microRNA-181s in HCC [184]. These results suggest a novel regulatory link between microRNA-181 family members, Wnt/beta-catenin signaling, and liver cancer stem cells and implies that molecular targeting of microRNA-181 or Wnt/beta-catenin signaling may eradicate hepatocellular carcinoma (HCC).

Studies have also recently explored whether specific microRNAs exist in hepatic cancer stem cells (CSCs) that are not expressed in normal hepatic stem cells by assessing the microRNA transcriptome of HCC specimens by small RNA deep sequencing [185]. It was found that miR-150, miR-155, and miR-223 were preferentially highly expressed in EpCAM+ HCC cells and their gene surrogates were associated with patient prognosis. Further studies showed that suppressing miR-155 resulted in reduction of EpCAM + HCC cells, reduced HCC tumorigenicity, and shortened overall survival and time to recurrence of HCC patients. Thus, miR-155 was highly elevated in EpCAM1 HCC cells and might serve as a molecular target to eradicate the EpCAM+ CSC population in human HCCs.

While EpCAM seems to be a positive marker of HCC CSCs, others have shown that HCC cells may also be positive for CD133 or CD90, indicating that these antigens are also features of cancer stem cells [186, 187]. 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 dedifferentiation 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.

5.7 Future Directions

5.7.1 Sequencing

Recently, a more comprehensive view of the genome has been made through the use of sequencing technology. We are now able to define specific mutations in the protein-coding region (exome), the whole genome, and various RNA transcripts. These approaches have led to the

Table 5.1 A summary of HCC DNA sequencing studies

Platform*	Sample size	Candidate driver genes	Study/year	References
Whole genome	147	ATM, CTNNB1, ARID1A, IGSF10, TP53,	Fujimoto et al. (2012)	[218]
Whole genome	88	CTNNBI, LRP1B TP53	Kan et al. (2013)	[22]
Whole genome	608	CTNNB1, TERT, TP53	Totoki et al. (2014)	[193]
Whole exome	149	ARID1A, AXIN, CTNNB1, RPS6KA3, TP53	Guichard et al. (2012)	[219]
Whole exome	11	TERT	Woo et al. (2014)	[190]
Whole exome	110	ARID1A, TP53	Huang et al. (2012)	[191]
Whole exome	87	CTNNB1, TP53	Cleary et al. (2013)	[188]
Whole exome	235	ALB, ARID1A, AXIN1, CTNNB1, TERT, TP53	Schulze et al. (2015)	[194]

*Manuscripts were selected based on the use of next-generation sequencing methods in human cohorts. Candidate driver genes are presented in alphabetical order and represent those genes found at greater than 10 % frequency in the noted study

discovery of novel genes in HCC. For example, whole exome sequencing has identified alterations of ARID1A, RPS6KA3, IRF2, NFE2L2-KEAP1, KMT2A in HCC [20, 188]. In addition, ARID2 has been implicated in HCV-associated HCC by whole exome sequencing, while CTNNB1 was found to have a pivotal role in HBV-HCC [189, 190]. This method has also been used to identify important genes associated with HCC metastasis, including CUL9, FGD6, KDM6A, AKAP4, and RNF139 [191].

The identification of genomic alterations in the full genome has also been attempted to understand the alterations occurring in noncoding regions and by structural rearrangements of the genome (Table 5.1). Several thousand somatic mutations and numerous chromosomal alterations were found by whole genome sequencing of a single HCV-HCC case by Totoki et al. In a study of mainly HBV-HCC, the JAK/STAT and WNT/Bcatenin pathways were found to be important drivers [192]. Recently, this work has been expanded in over 500 liver cancer cases, uncovering 30 candidate driver genes and 11 core pathways including metabolic enzymes, chromatin remodelers, and TERT as a central and ancestry-independent node in HCC [193]. In addition, DNA mismatch repair genes and chromatin regulators, including ARID1A, ARID2, and MLL3 were mutated in a study of HCC including both HBV and HCV patients [21]. In an exome sequencing study by Schulze et al., TERT promoter mutations were identified as early events in HCC, while TP53, CTNNB1, CDKN2A and FGF family members were related to more advanced HCC stages [194]. Whole genome sequencing has also allowed for the identification of viral integration sites caused by the DNA virus, HBV, and genomic aberrations that occur near those sites. Important integration sites include TERT, MLL4, FN1, and CCNE1 [22, 195]. Retrotransposon insertions and repetitive sequences have also been explored by whole genome sequencing. Two long interspersed nuclear element-mediated somatic changes in MCC and ST18 have recently been described in HCC [196].

RNA sequencing, meanwhile, provides an extension of transcriptomic profiling by allowing for the assessment of translocation and inversions of transcripts, noncoding RNAs, and splicing events. Splicing variants for several genes have been reported in HCC including TCF4, KLF6, p73, and LLGL1 [197]. RNA editing events have also been explored by this methodology and have identified a gain of function activity in the AZIN1 gene in HCC along with RNA editing roles of BLCAP [198–200]. These studies are rather small in sample number and await further exploration in larger datasets.

5.7.2 Circulating Tumor Cells

Although hepatic resection and liver transplantation are the main modalities of curative HCC treatment, approximately 40 % of hepatectomy patients and 10 % of transplant patients develop postoperative recurrences. One factor that is thought to underlie this outcome is the presence of circulating tumor cells (CTCs) which may be released from the primary tumor or metastatic lesions. In the last decade, effort has been placed on identifying and improving technology and methods to detect CTCs, understand their role in tumor biology and usefulness as tumor biomarkers. These include enrichment methods based on physical characteristics and/or immunological markers, microfilters, density gradient centrifugation, and microfluidic chips [201, 202]. Once enriched and isolated, various methods are used to characterize CTCs including nucleic acid analysis, cytometric analysis, and functional analysis. The characterization and enumeration of CTCs may be a significant advance in our understanding of tumor heterogeneity, patient stratification for treatment or treatment response, and risk of relapse.

Table 5.2 A summary of HCC integrated omics studies

Integrated platforms*	Sample size	Candidates/signatures	Study/year	References
Double platform integration				
Transcriptome + Metabolomics	356	SCD1 (lipid signature)	Budhu et al. (2013)	[210]
Transcriptome + aCGH	61	Metastasis genes	Roessler et al. (2015)	[220]
Transcriptome + aCGH	76	PROSC, SH2D4A, and SORBS3 (tumor suppressors)	Roessler et al. (2012)	[207]
Transcriptome + aCGH	380	YY1AP1 (metastasis/stem cell)	Zhao et al. (2015)	[208]
miRNA + mRNA	100	miR-148-ACVR1/BMP	Li et al. (2015)	[209]
RNA Seq + DNA Seq	2	BLCAP (RNA editing)	Hu et al. (2015)	[199]
Methylation + Transcriptome	71	SMPD3, NEFH (tumor suppressors)	Revill et al. (2013)	[213]
Methylation + Transcriptome	128	CFH, MYRIP, PSRC1, MRE11A and MYO1E (tumor recurrence)	Yang et al. (2011)	[214]
Triple platform integration				
RNA Seq + DNA Seq + SNP	174	TTK (mitotic checkpoint)	Miao et al. (2014)	[217]
Methylation + Transcriptome + aCGH	63	PER3, IGFALS, protein Z (tumor suppressors)	Neumann et al. (2012)	[216]
Methylation + Transcriptome + SNP array	49	COL1A1 (survival)	Hayashi et al. (2014)	[215]

*Manuscripts were selected based on the integration of two or more omic platforms and the use of human cohorts

A few studies have been published regarding CTC detection and characterization in HCC. The clinical usefulness of CTC counts was reported in a preliminary study by Vona et al. in 44 HCC patients showing association of CTCs with later disease stage and shorter survival [203]. Detection of CD45(−)CD90(+)CD44(+) or EpCAM(+) cells have also been employed to predict HCC recurrence and metastasis [204–206]. Current strategies are focused on further characterizing CTCs and understanding their modes of release and circulation in order to prevent or reduce the risk of recurrence, metastasis, and improve survival rates.

Our ability to define specific CTCs by single markers or overlap of specific markers will also aid in understanding the pools of CTCs that may be present in a given tumor or tumor subtype that could allow us to better identify and stratify HCC patients for effective treatment, etc. This may also lead to strategies for targeting and/or eliminating CTCs in order to prolong patient survival. Although the amount of data and evidence concerning CTCs are growing in the HCC field, currently there is still a lack of definitive evidence that the detected cells are specific to HCC, capable of stem-like abilities and initiate metastasis or recurrence. In addition, current CTC capture techniques will need to be improved in order to increase the purity of isolated cells and their yield. Overall, CTCs represent an important new strategy to identify markers for patient relapse and poor survival and may be targetable populations to reduce these outcomes.

5.7.3 Data Integration

While array-based technologies have allowed us to define molecular alterations at various levels of the genome, it is important to note that these factors do not act on their own, but rather, make up complex networks that span several levels of genomic and genetic signaling. In this vein, it is important for us to be able to understand how these factors interact and/or are affected by one another to produce the final phenotype that is observed. Thus, many researchers involved in high-throughput genomics have begun to explore signaling networks, rather than single molecules, as methods of defining important molecular nodes and drivers of HCC. Such integrated approaches are thought to be an improved strategy of resolving the important and key molecules that cause HCC and allow it to progress (Table 5.2).

We have also recently used integrative approaches to identify HCC driver genes. For example, we have combined high-resolution, array-based comparative genomic hybridization, and transcriptome analysis of HCC samples to identify and validate a 10-gene signature associated with chromosome 8p loss and poor outcome [207]. Functional studies demonstrated that three gene products among the 10-gene signature have tumor suppressive properties. Integrated genomics has also recently been used to identify YY1AP1 as an oncogenic driver in stem-like HCC [208]. In

an integration study of miRNA and mRNA profiles, the miR148a-ACVR1/BMP circuit was useful in defining a stem cell-like aggressive subtype of HCC [209]. Metabolite and mRNA profiles have also been integrated to define key signaling events that can alter the fitness of EpCAM+ AFP + HCC cancer stem cells [210]. Our analysis revealed tumor-specific and stem cell-like-specific metabolites linked to patient survival along with correlating significant genes in the stem cell-like tumor subgroup. In particular, stearoyl CoA desaturase (SCD), a key enzyme involved in fatty acid biosynthesis, and its related metabolites were highly elevated in stem cell-like HCC and are associated with HCC survival and may functionally contribute to HCC stemness and aggressiveness. We have also recently compared and contrasted global metabolic profiles between liver, breast, and pancreatic cancer tissues and found that metabolites are principally unique to each tissue and cancer type. Thus, metabolic profiling could be applied as cancer classification tools to differentiate tumors based on tissue of origin [211].

To aid in the integration of multiple omics data, we have proposed an integrative subgraph mining approach, called iSubgraph to discover patterns of miRNA-gene networks which could be used for patient stratification in HCC [212]. This algorithm could detect cooperative regulation of miRNAs and genes with highly stable class predictions. The HCC subgroups identified by the algorithm have different survival characteristics with key roles of specific genes in HCC subgroups. Thus, our method can integrate various omics data derived from different platforms and with different dynamic scales to better define molecular tumor subtypes.

Integrative genomic analysis of genome-wide methylation and gene expression data identified possible key targets in HCC. Recently, using this method, the tumor suppressive roles of SMPD3 and NEFH have been demonstrated in HCC [213]. Evidence was provided that SMPD3 is a potent tumor suppressor gene that could affect tumor aggressiveness, while a reduced level of SMPD3 is an independent prognostic factor for early recurrence of HCC. This method was also used to identify genes associated with HCC recurrence, including CFH, MYRIP, PSRC1, MRE11A, and MYO1E [214]. Triple-combination array analysis of expression arrays, SNP array, and methylation array successfully identified COL1A1 as a candidate survival-related gene in HCCs. Epigenetic downregulation of COL1A1 mRNA expression might have a role as a prognostic biomarker of HCC [215]. A combination of genome-wide methylation, array CGH, and gene expression was also used to identify PER3, IGFALS, protein Z as HCC tumor suppressors [216]. Whole genome sequencing has been integrated with transcriptome sequencing and SNP genotyping to identify a dual-specificity protein kinase, TTK as a prognostic indicator of HCC [217].

Integration among various levels of omics signaling may help to further define the key players that promote HCC and affect its progression. For clinical application, it is also useful to integrate omic information with current clinical triage methods, including tumor staging and pathology, to further refine patients into risk groups. This combined information can then be applied to stratify patients for the most appropriate and likely to be most effective treatment regimens. This strategy underlies the topic of precision medicine, whereby a more individualistic approach based on the combination of science and medicine is used to manage patient care.

5.8 Summary

The advent of array technology has provided a high-throughput methodology to assess the genome-wide changes that occur during hepatocarcinogenesis and its progression. 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.

Arrays 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 arrays, 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 technique such as laser capture microdissection and automation has 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 posttranscriptionally. Therefore, elements such as protein localization and modification are important elements to be included in HCC profiling. Difficulties in data comparison and integration must also be addressed which ensues from the use of multiple array 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 array statistical analysis and reporting such as those established by the International Microarrays Gene Expression

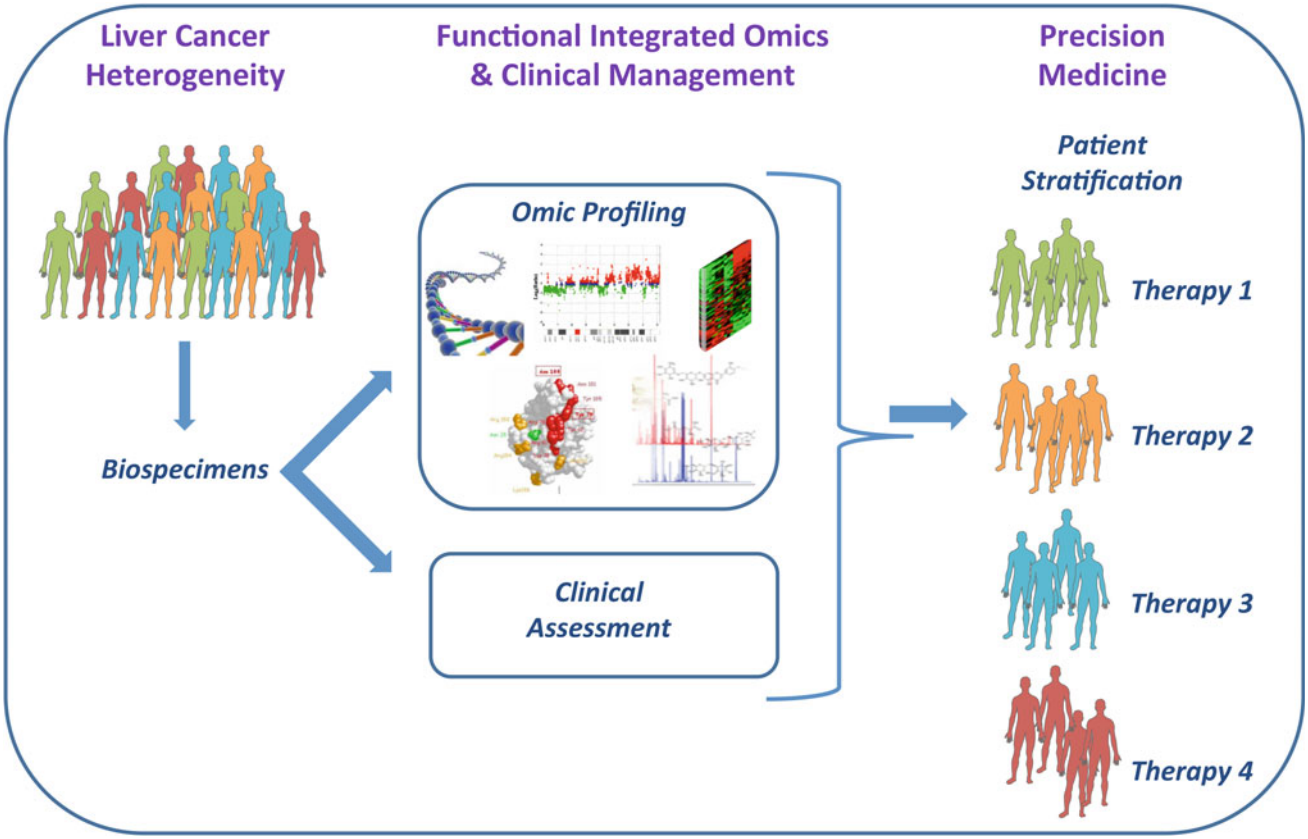

Fig. 5.1 Functional and integrated omic profiling for biomarker identification, validation, and clinical utility. Widescreen genomic profiling of hepatocellular carcinoma (HCC) has identified multiple biomarkers on the gene, protein, and genomic scale. These biomarkers are useful for understanding HCC biology and clinical application. The mechanistic and clinical information gleaned from genomic profiling studies can be combined using computational strategies to identify promising novel therapeutic markers for diagnosis, treatment, and prognosis of HCC. Such methods will allow progression toward precision medicine encompassing new and selective therapeutics and preventative therapy

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. Lastly, each array can only provide information concerning the targets that are included in that array. Thus, integrative analysis of multiple platforms may be required 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 also be required to make such assessments. Recently, systems have been developed 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 a cost-effective and accurate manner and may allow for ease in cross-platform-type analyses since an enormous multilevel dataset can be achieved with a relatively small amount of the same starting material. Overall, integrating global molecular profiling data along with mechanistic/functional studies may improve the diagnosis, treatment, and prognosis of HCC patients.

Although multiple publications have identified and validated diagnostic and/or prognostic HCC markers, 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 noninvasive 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 (Fig. 5.1). The HCC-related genomic expression studies presented in this chapter along with future studies and advances in array 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 patients. We are now at the brink of clinically implementing biomarkers identified from global array profiling to improve HCC diagnosis, treatment, and outcome.

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Genomic Signatures of Risk Factors and Molecular Identification of HCC Subtypes

6

Jean-Charles Nault and Jessica Zucman-Rossi

Contents

The main risk factors of HCC (infections by hepatitis B or C viruses, high alcohol consumption, metabolic genetic diseases or obesity) predispose to chronic liver disease leading to cirrhosis development, a major pre-cancerous stage for cancer initiation [1, 2]. HCCs occur in 90 % of the cases in cirrhotic patients and its occurrence increased with the severity of cirrhosis. However, in 10 % of the remaining cases, HCC are discovered in non-cirrhotic patients mainly in HCC related to metabolic syndrome, HBV infection or without known etiology [3]. In these latter patients, we can hypothesize that exposure to additional risk factors and/or genetic predisposition could contribute significantly to the development of HCC [4]. Overall, specific risk factors can influence the process of hepatocarcinogenesis and this is particularly important for environmental factors [5].

6.1 Genomic Alterations Related to Early Hepatocarcinogenesis

HCC, as other solid tumors, is a disease of the genome [6]. Their development results from the accumulation of genetic and epigenetic alterations in hepatocytes that gives a selective proliferative advantage [5, 7] (Fig. 6.1). As in all cells of the body, these mutations are accumulated randomly during life since birth, however, hepatocytes are also major targets for genotoxic agents because of the key role of liver in detoxification [5]. Exposure to genotoxic agents damages the genome of hepatocytes and increases the number of somatic mutations accumulated in the cells [4, 8]. In most of the cases, random mutations occur in intergenic regions and have no functional consequences on cell biology. However, when mutations occur within a cancer driver gene it can initiate or promote [9] the mechanism of tumorigenesis. As a result of this process, in each HCC, a mean number of 40 mutations altering the function of different genes are identified [10–12]. Moreover, the pattern of mutations could give

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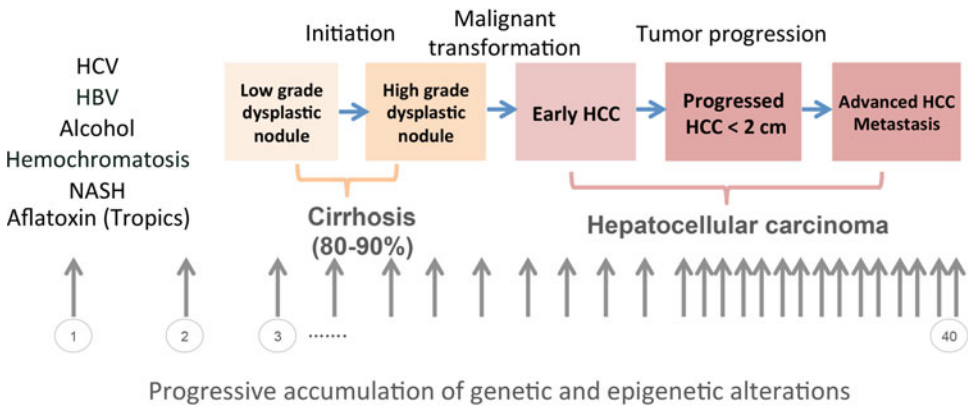

Fig. 6.1 Hepatocarcinogenesis is a multistep process with a progressive accumulation of genomic alterations in tumor hepatocytes. In each HCC, a mean number of 40 functional damaging mutations are accumulated

several clues to understand the underlying mutagenic process and identify new risk factors of HCC development [9, 13].

6.1.1 Exposure to Genotoxic Agents

Exposure to Aflatoxin B1 (AFB1) in subtropical countries is a prototype of genotoxicity in the liver [14]. Aspergillus Flavus fungi, that colonize food in subtropical countries, produce Aflatoxin B1 [14]. AFB1 metabolites are genotoxic accumulated in the liver, they form adducts to DNA and induce nucleotide transversions with a recurrent specific mutation identified in TP53 gene at codon 249 [15, 16]. AFB1 cooperates with HBV infection and GSTM1 polymorphism to increase the risk of HCC development [17, 18]. Recent sequencing of HCC genome identified a specific pattern of mutation thorough the genome related to AFB1 exposure in HCC developed in patients from African subtropical area [11]. This pattern of mutations is characterized by C>A transversions with a transcriptional DNA strand bias (Signature 24). Aristocholic acid is the product of Aristolochia plants used in Chinese herbal remedies that induces nephrotoxicity but also urothelial carcinoma of the upper urinary tract [19]. Moreover, specific mutational signature A:T-to-T:A transversions with a bias on the non transcribed strand have been identified in urothelial carcinomas of the upper urinary tract but also in rare cases of HCC. It links exposure to aristocholic acid with HCC development [20, 21].

Analysis of the mutational signature in HCC showed that accumulation of gene mutation is also related to the age of the patients suggesting that it is a progressive process during life [11]. During the development of chronic hepatitis and cirrhosis, hepatocytes are exposed to oxidative stress, inflammation and cell senescence whatever the risk factor [22]. These processes can also induce a "genotoxic stress" and promote the accumulation of genomic defects [8]. Also, tobacco smoking, which is recurrently identified as a risk factor participating to HCC development, could induce specific mutational signature in the genome of HCC [11]. Finally, genotoxic agents at the origin of specific mutational signature remain to be identified and future epidemiological studies combined to molecular analysis of the tumor are promising to help to better understand the contribution of various environmental exposures in HCC development.

6.1.2 Viral Infections Inducing Insertional Mutagenesis in Hepatocytes

HBV is a DNA virus with a genome of 3,300 bases that plays a key role in hepatocarcinogenesis [23]. HBV infection and expression of viral proteins like Hbx increase chromosome instability and promote cell proliferation. HBV integrations into the genome of infected hepatocytes can activate or inactive the function of the targeted genes by insertions, deletions or rearrangements [24, 25]. This phenomenon known as insertional mutagenesis promotes clonal proliferation of tumor hepatocytes and is supposed to be an early genetic event in viral induced tumorogenesis [25, 26]. Insertional mutagenesis is a direct viral oncogenic mechanism that explains the occurrence of HCC on normal liver in patients with chronic HBV infection [27]. Recently, using next generation sequencing technics, a large number of insertion sites were identified [28, 29]. Several insertion sites of HBV were recurrent in HCC and the most frequent events occurred in TERT promoter activating telomerase or in MLL4 [28, 30].

Recently, sequencing a series of HCC revealed clonal insertions of the adeno-associated virus type 2 (AAV2) in 11 tumors mainly developed in young patients without significant liver fibrosis and known risk factors [31]. AAV2 is a DNA virus that persists in a latent form inserted in human DNA. It was considered previously as a non-pathogenic [32, 33]. The viral insertions were identified in HCC within cancer driver genes and lead to activate their expression using an insertional viral mutagenesis similar to that observed with HBV in HCC. Common genes are targeted by HBV and AAV2 insertions at TERT promoter, MLL4, CCNA2 and

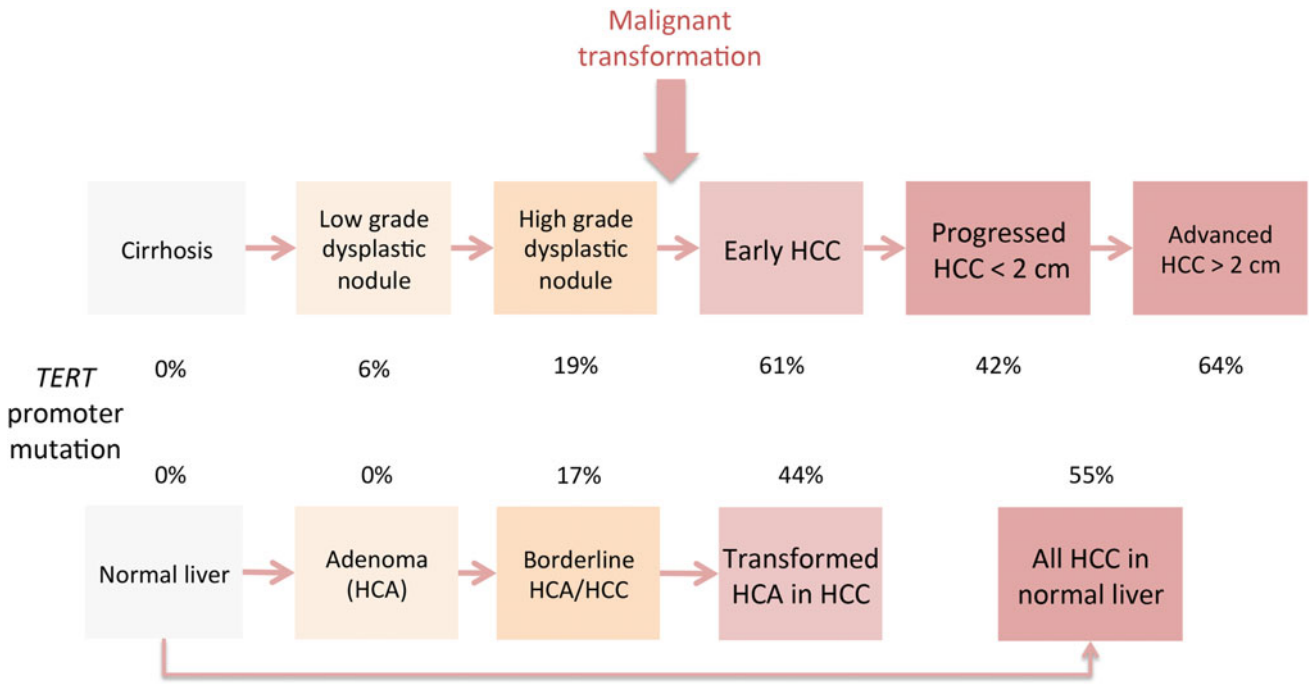

Fig. 6.2 Somatic TERT promoter is the earliest recurrent somatic mutation in HCC developed in cirrhotic and non-cirrhotic liver. Adapted from Nault et al. [45, 46], Pilati et al. [53]

CCND1 activating cyclin A2 and E1 [28, 31, 34]. These events of AAV2 insertion in oncogenes promoting HCC development are exceptional since HCC developed in normal liver are very rare. In contrast, AAV2 asymptomatic infection is highly frequent in the general population since 60 % of the individual are infected by AAV2 during life [35]. This paradox between a frequent viral infection and the development of a rare subtype of cancer is also found with EBV infection inducing Burkitt lymphoma or in Merkel Cell Carcinoma, a rare aggressive skin cancer associated with Merkel cell polyomavirus (MCV) infection [35, 36].

Finally, both HBV and AAV2 are "genotoxic" DNA viruses in hepatocytes since they can modify the genome of the host cells altering cancer driver genes [37, 38]. Up to now, AAV2 is the 8th virus associated with cancer development after HBV, HCV, HPV, MCV, EBV, HTLV1 and HHV8 [31, 35]. The reasons why only a small subset of the individuals is developing tumors after this infection remain to be elucidated.

6.1.3 Somatic Mutations in the Telomerase Promoter Is the Earliest Recurrent Genomic Alteration

Malignant transformation of hepatocytes occurring in cirrhosis is a multistep process with defined histological sequence of lesions and the successive occurrence of low-grade dysplastic nodules (LGDN), then high grade dysplastic nodules (HGDN) that are at higher risk of malignant transformation in early HCC that progress in small and progressed HCC and advanced HCC [39, 40]. Telomerase complex that control the length of the telomeres, the repeated sequences that protect chromosome extremities from erosion, play a key role in cirrhosis pathogenesis and malignant transformation of hepatocytes [41–43] Reexpression of telomerase is observed in more than 90 % of HCC and allow an unrestrained proliferation of tumor hepatocytes. [44]. Activation of telomerase, by somatic TERT promoter mutation, is the earliest and the most frequent somatic genomic alteration occurring in the mechanism of HCC development occurring in cirrhotic or non-cirrhotic liver [45, 46]. TERT promoter mutations were first identified in 70 % of melanoma [47–49]. In HCC, mutations occur at two hot spots in TERT promoter located 126 and 144 nucleotides upstream the traduction initiation codon of the telomerase gene [50]. These mutations generate a "CCGGAA" sequence, a binding site for transcription factors of the *ETS* family as GABP that activate TERT transcription [51, 52].

TERT promoter mutations are not observed in cirrhosis, but its frequency increase progressively during hepatocarcinogenesis since they are identified in 6 % of low-grade dysplastic nodule, in 19 % of high grade dysplastic nodules and in around 60 % of early, small and progressed HCC [46] (Fig. 6.2). TERT promoter mutations are also identified in HCC developed in non-cirrhotic liver. Moreover, it is a

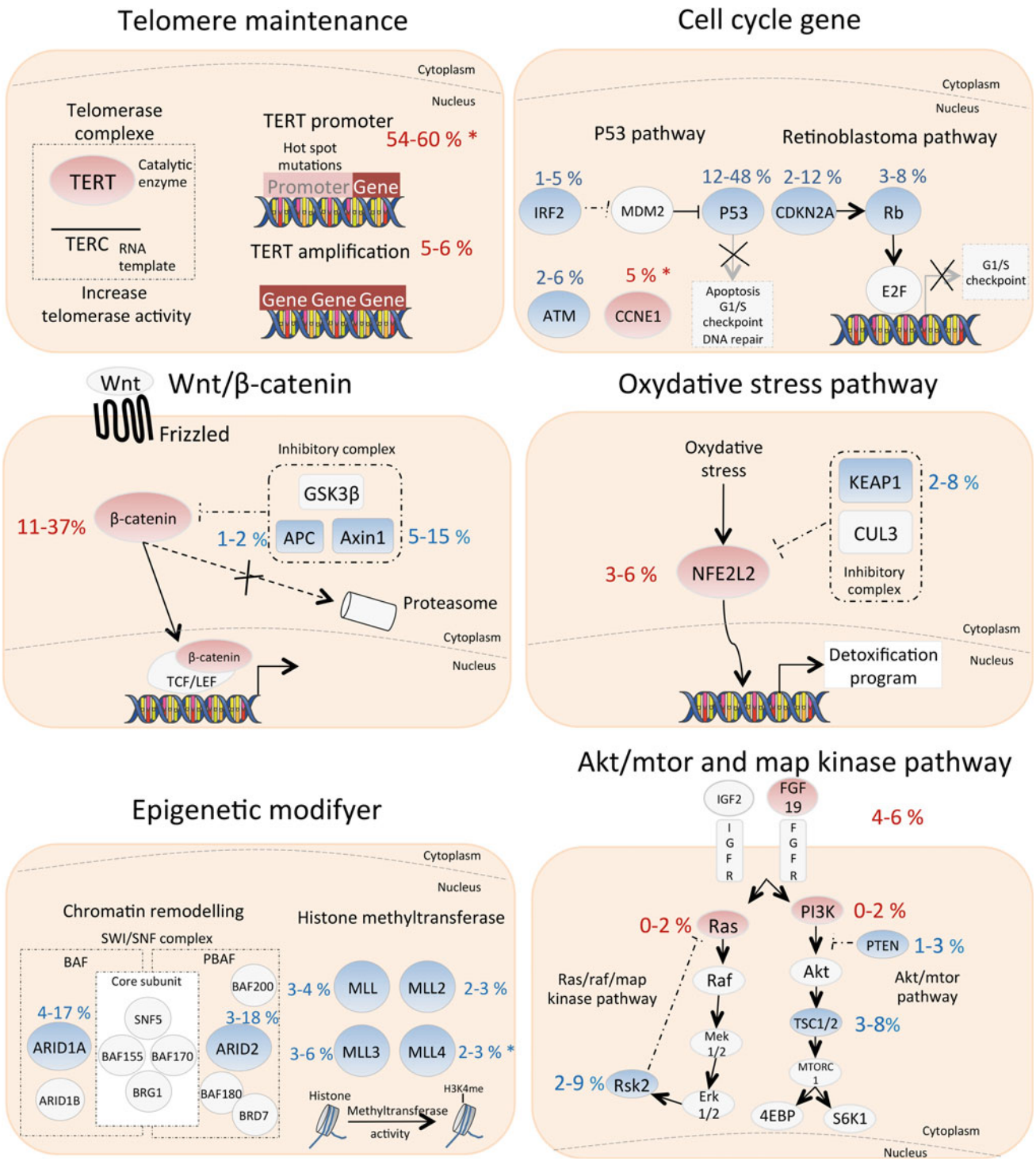

Fig. 6.3 Major pathways altered in HCC. Frequency of gene mutations are indicated in oncogenes (*gene in red*) or tumor suppressor genes (*in blue*) according to Guichard et al., [10], Totoki et al. [12], Schulze et al. [11], Zucman-Rossi {Zucman-Rossi 2015 #4499}

major genomic event, in association with activating mutations of CTNNB1, involved in the malignant transformation of hepatocellular adenoma (HCA) in HCC [45, 53, 54] (Fig. 6.2).

6.2 Genomic Alterations in Progressed HCC

Activation of telomerase, by TERT promoter mutation, is the earliest and the most frequent alteration occurring in the mechanism of HCC development (60 % of mutations in progressed HCC) [45], then, additional mutations are accumulated during HCC progression. Recurrent activating mutations of *CTNNB1* (11–37 %) activating ß-catenin are the second alterations the most commonly observed in HCC frequently associated with alcohol intake and HCV infection (Figs. 6.3 and 6.4) [11, 55, 56]. Inactivating mutations in *TP53* are also highly frequent (20–52 %), more predominant in HBV-related HCC and associated with poor prognosis [10, 15, 57, 58]. Notably, alterations in *CTNNB1* and *TP53* are usually exclusive from each other; they define two different subgroups of HCC with different genes dysregulated at the genomic level [11, 59, 60]. Several genes encoding chromatin remodeling factors and histone methyltransferase are also frequently mutated in HCC [5]. Among them, *ARID1A* and *ARID2* are the genes the most recurrently inactivated in HCC cohorts around the world (8–17 %) [10–12, 61]. Somatic mutations in several additional cancer drivers have been also identified (see Fig. 6.3).

Recurrent copy number alterations (CNA) leading to losses and gains of large chromosome regions were identified [60, 62]. Recurrent homozygous deletions inactivating tumor suppressor genes or recurrent focal amplifications activating oncogenes were described (included in Fig. 6.3) [10]. Integrating CNA and frequent mutations in 161 putative cancer driver genes, pointed out frequent alterations of major oncogenic pathways involved in telomere maintenance, Wnt signaling, PI3 K/mTOR pathway, p53 pathway, MAP kinase pathway, hepatic differentiation, epigenetic regulation, chromatin remodeling, oxidative stress, JAK/STAT pathway, and TGFß signaling [11, 12]. Nevertheless, noticeable differences are observed among HCC cohorts, reflecting heterogeneity related to geographical and risk factor distributions (Fig. 6.4) [11, 63]. The identification of the major driver genes recurrently mutated in progressed

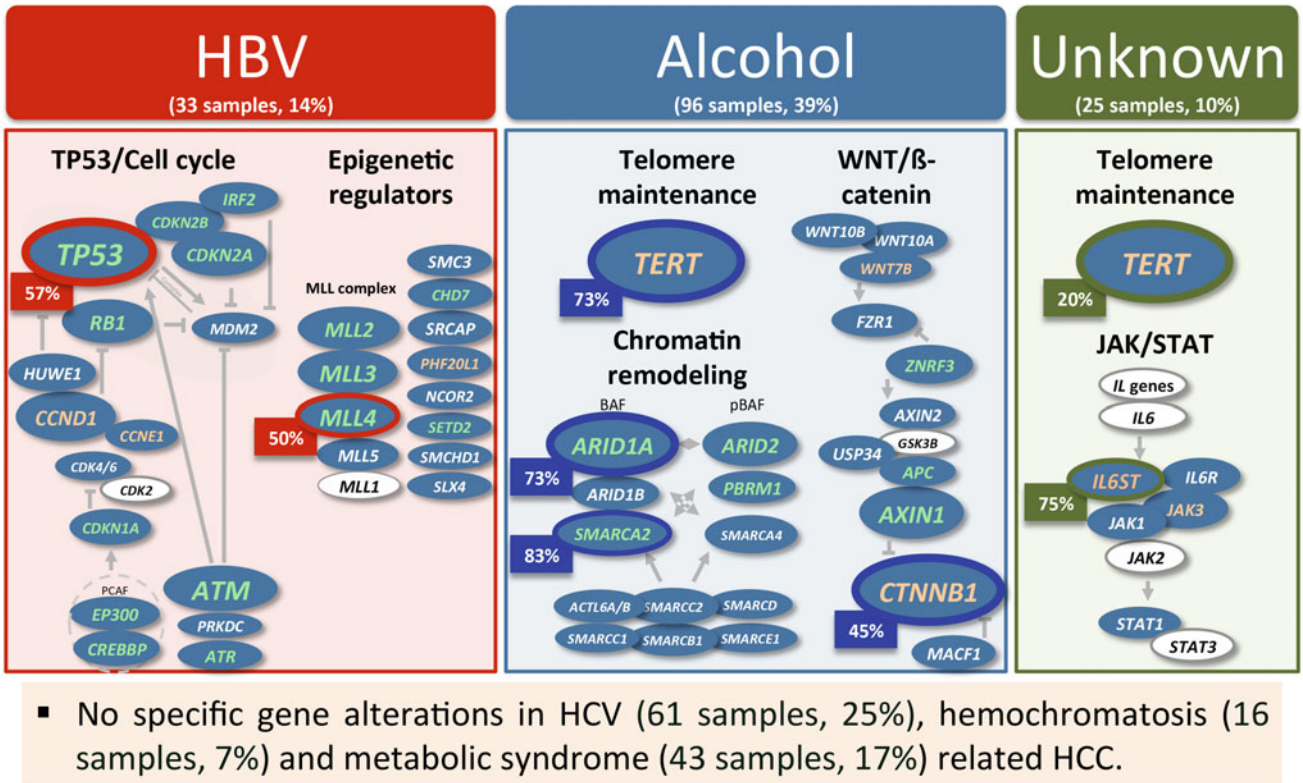

Fig. 6.4 Enrichment in gene mutations according to the different risk factors. HBV-related tumors are enriched in P53 and MLL4 alterations; alcohol related tumors in CTNNB1, TERT promoter, ARID1A and SMARA2 mutations; HCC with unknown etiology more frequently associated with IL6ST mutations whereas no specific pattern of gene mutations were identified in HCV, hemochromatosis and metabolic syndrome related HCC [11]

HCC is the first step to develop biotherapy tailored to genomic alterations [7, 64].

Conclusion: HCC is a heterogenous disease and the natural history of HCC development is related to various molecular subtype of HCC and genotoxic exposure. Molecular diversity of HCC is at least partly driven by the different risk factors and the underlining chronic liver disease that predispose to tumor development. The development of next generation sequencing offers a unique opportunity to better understand the relationship between exposure to risk factors and the mechanisms of carcinogenesis in the liver.

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MicroRNAs and Hepatocellular Carcinoma

7

Aldo Cavallini

Abbreviations

AAV	Adenoviral vector
ADAM9	ADAM metallopeptidase domain 9
AFP	a-fetoprotein
ALT	Allograft transplantation
AMO	Anti-miRNA antisense oligomer
CEA	Carcinoembryonic antigen
DGCR8	DiGeorge syndrome chromosomal region 8
Dicer	Ribonuclease III
Drosha	Double-stranded-RNA-binding protein
5-FU	5-fluorouracil
ITBLs	Ischemic-type biliary lesions
LT	Liver transplantation
miRNA	MicroRNA
mRNA	RNA messenger
OLT	Orthotopic liver transplantation
OncomiR	miRNA with oncogene role
OT	Operational tolerance
pre-miRNA	Preliminary miRNA
pri-miRNA	Primary miRNA
RISC	Multiprotein RNA induced-silencing complex
RNAi	RNA-mediated interference
siRNA	Small interfering RNA
snRNA	Small noncoding RNA
TACE	Transcatheter arterial chemoembolization
TS miR	Tumor Suppressor miRNA
UTR	Untranslated region
XPO5	Exportin 5
ZEN	*N*,*N*-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine

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Contents

7.1 Introduction

Genomic studies have demonstrated that many portions of the human genome do not encode conventional protein-coding genes but encode biologically active noncoding RNA species. One class of such small noncoding RNA is microRNA (miRNA), comprised of a group of well-conserved, small RNA molecules (21–23 nucleotides) that can up- or down-regulate gene expression in normal and abnormal cellular activities by base pairing with 3′ untranslated regions (3′ UTRs) of target messenger RNA (mRNA) [1].

In addition, miRNAs can function both as tumor suppressor (TS-miR) or oncogene (oncomiR) based on the combination of different target RNA messengers (mRNAs). When a miRNA prevents cancer formation in healthy cells, this miRNA is called TS-miRNA. The miRNAs that are increased in neoplastic tissues, that usually promote tumor development by negatively inhibiting tumor suppressor genes and/or genes that control cell differentiation or apoptosis, are called oncomiRs.

miRNAs were discovered for the first time in 1993 by Lee's group [2] and, after two decades, thousands of miRNAs have been identified in human.

About 3 % of genes encode 2000 different miRNAs in human, and have been shown to play critical roles in normal cellular functions, such as growth, development, differentiation, and reproduction [3–6].

Since miRNAs affect the development of tumors, including hepatocellular carcinoma (HCC), via dysregulation of their biogenesis and gene expression, several experimental studies have discovered increasing numbers of aberrantly expressed miRNAs which are involved in molecular mechanism of HCC progression.

In this chapter, we focus on clinical applications of miRNAs in HCC and on recent advances of miRNAs as drugs.

7.2 miRNA Biogenesis and Action

The generation of mature miRNAs is a multistep process that starts with the initial transcription of their genes by RNA polymerase II. In the nucleus RNA polymerase II transcribes the primary miRNA (pri-miRNA), a double strand of about 1000 base pair (bp). Subsequently, pri-miRNAs are processed to an miRNA precursor (pre-miRNA) of 60–100 bp by a protein complex (Drosha–DGCR8). Then, pre-miRNAs are transported from the nucleus to the cytoplasm by exportin-5 (XPO-5) and further cleaved (21–24 bp) by another protein complex (Dicer). These two strands are separated and one strand is degraded, while the second strand, that represents the mature strand, binds to RNA-induced silencing complex (RISC). Finally, mature single-stranded miRNA by RISC recognizes the target mRNA. The miRNA–mRNA interaction usually causes translational repression by imperfect pairing with mRNA and/or mRNA cleavage by perfect pairing with the target mRNA. In both cases the final result is the reduction of the protein output.

For more details see paper by Wahid et al. [7].

7.3 Up- and Down-Regulated miRNAs in HCC

The first association between miRNAs and cancer was identified by Croce et al. [8] when the miR-15a-16-1cluster was implicated as a putative tumor suppressor gene mapping to chr 13q14, a small genomic region frequently deleted or translocated in chronic lymphocytic leukemia.

Subsequently, the abnormal expression of miRNAs has been demonstrated in a variety of cancers considering that in biological processes of a tumor are involved different pathways, such as proliferation, apoptosis, invasion, migration, and metastasis.

An investigation has also added indirect support that miRNA changes are causal, rather than consequential, of cellular transformation [9].

It is well known that cancer is associated with very complex genetic alternations in oncogenes and tumor suppressors, and several evidences suggest that also miRNAs can function as oncogenes or tumor suppressors [10].

In oncogenesis, some miRNAs expression is decreased in cancerous cells and these types of miRNAs are considered tumor suppressor genes. Tumor suppressor miRNAs role is usually to prevent tumor development by negatively inhibiting oncogenes and/or genes that control several metabolic pathways, such as proliferation or apoptosis. On the contrary, those miRNAs whose expression is increased in tumors may be considered as oncogenes. These oncogene miRNAs, called "oncomirs," usually promote tumor

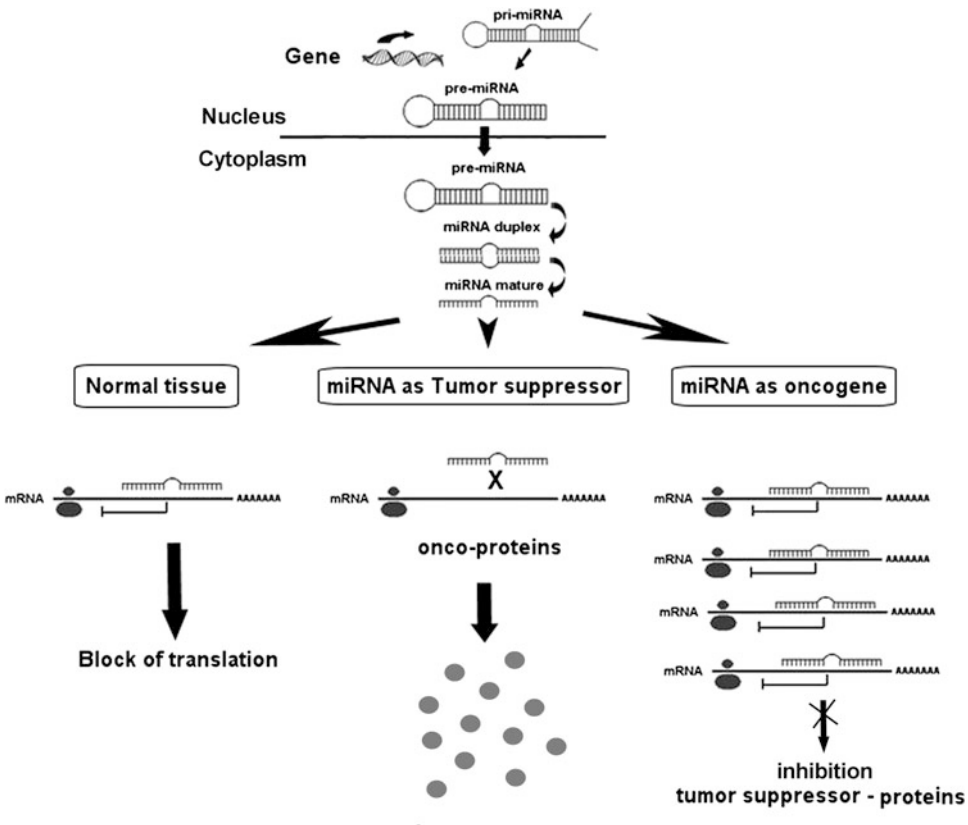

Fig. 7.1 Biogenesis of a miRNA as oncogene or tumor suppressor

development by negatively inhibiting tumor suppressor genes. Many of these miRNAs have been found that are significantly over-expressed in different cancers, including HCC [11, 12].

The mechanisms of both tumor suppressor miRNA and oncomir are depicted in Fig. 7.1.

Altered microRNA expression are differentially expressed in diverse human liver diseases, from chronic liver diseases with different etiology to HCC through cirrhosis [13]

Here, we address on miRNA profile in HCC. The results obtained from numerous studies show a total of 80 down-regulated and 79 up-regulated miRNAs (Table 7.1).

Moreover, experimental data by in vitro and in animal models have also identified the mRNA target for each miRNA. Discovery of the miRNA role in various human pathological processes has further shed light to the possible applications of the miRNAs in molecular diagnostics, prognostic and therapy for HCC.

7.4 Circulating miRNAs as Diagnostic or Prognostic Tool in HCC

Studies suggest that miRNAs are not only localized within the cell or in tissue environmental, but are also present in circulation. Extracellular circulating miRNAs can be found in lipid or lipoprotein complexes or packed into microvesicles, known as exosomes. These miRNAs provide an association between their serum levels and specific organ dysfunction. Therefore, this suggests that circulating miRNAs may represent a new class of prognostic and diagnostic cancer biomarkers [115–117].

In addition, serum-derived miRNA biomarkers would be advantageous compared with the examination of HCC tumor tissue due to the noninvasive nature of the sampling and the possibility of repeated sampling. Moreover, since many studies have demonstrated that miRNA expression profiles in HCC and non-tumor tissue are significantly different, serum miRNAs can reflect the level of tissue miRNAs and thus potentially be used as HCC markers.

With the aim of identifying new and specific biomarkers for HCC, Koberle et al. [118] report a prognostic potential of miR-1 and -122 in sera of HCC patients.

Serum miR-1 and -122 concentrations did not differ significantly between patients with HCC and liver cirrhosis, whereas both miRNAs were associated with overall survival (OS) in HCC patients. miR-1 serum levels did not correlate with clinical chemistry liver parameters, whereas serum miR-122 strongly correlated with the serum ALT, AST, and GGT levels.

The explanation is that miR-122 is a liver-specific, multifunctional RNA that controls several metabolic pathways,

Table 7.1 Up- and down-regulated miRNAs in HCC

Up-regulated				Down-regulated			
miRNA	Ref.	miRNA	Ref.	miRNA	Ref.	miRNA	Ref.
miR-9-3p	[14]	miR-210	[42]	Let-7a	[64]	miR-152	[87]
miR-10a	[15]	miR-213	[21]	Let-7b	[83]	miR-181	[46]
miR-10b	[16]	miR-216a	[43]	Let-7c	[65]	miR-185	[17]
miR-16	[17]	miR217	[44]	Let-7d	[64]	miR-194	[17]
miR-17-5p	[18]	miR-221	[45]	Let-7e	[46]	miR-195	[88]
miR-18a	[19]	miR-222	[28]	Let-7f-1	[64]	miR-198	[30]
miR-19b	[20]	miR-224	[47]	Let-7g	[66]	miR-199a-3p	[89]
miR-20a	[20]	miR-294	[21]	miR-1	[67]	miR-199a-5p	[90]
miR-21	[21]	miR-301a	[48]	miR-7	[68]	miR-199b-3p	[91]
miR-22	[22]	miR-324	[14]	miR-10a	[15]	miR-200a	[92]
miR-23a	[23]	miR-362	[28]	miR-10b	[69]	miR-200b	[93]
miR-24	[17]	miR-373	[49]	miR-15a	[70]	miR-200c	[94]
miR-25	[24]	miR-374	[14]	miR-21	[51]	miR-203	[95]
miR-26a	[25]	miR-376a	[21]	miR-26a/b	[95]	MiR-206	[96]
miR-27a	[20]	miR-378	[28]	miR-29a	[71]	miR-214	[97]
miR-30d	[26]	miR-382	[28]	miR-29b	[72]	miR-215	[27]
[miR-33	[27]	miR-423	[51]	miR-29c	[73]	miR-219-5p	[98]
miR-92-3p	[20]	miR-429	[63]	miR-34a	[74]	miR-223	[99]
miR-92-5p	[24]	miR-485-3p	[52]	miR-99a	[75]	miR-224	[100]
miR-93	[28]	miR-490-3p	[53]	miR-100	[76]	miR-292-3p	[94]
miR-96	[29]	miR-491	[28]	miR-101	[77]	miR-338	[17]
miR-100	[30]	miR-494	[54]	miR-122a	[78]	miR-365	[28]
miR-106b	[31]	miR-495	[52]	miR-124a-3p	[79]	miR-363-3p	[101]
miR-107	[32]	miR-500	[50]	miR-125a-5p	[80]	miR-375	[102]
miR-127-3p	[28]	miR-515-3p	[55]	miR-125b	[80]	miR-376a	[103]
miR-130b	[33]	miR-515-5p	[55]	miR-126-3p	[17]	miR-422a	[28]
miR-132	[46]	miR-517a	[56]	miR-128	[17]	miR-422b	[28]
miR-135a	[34]	miR-518a-3p	[55]	miR-130a	[17]	miR-424	[28]
miR-137	[35]	miR-519d	[58]	miR-132	[46]	miR-429	[104]
miR-143	[36]	miR-520f	[55]	miR-139-5p	[81]	miR-449	[105]
miR-151	[37]	miR-525-3p	[55]	miR-140-5p	[82]	miR-450a	[106]
miR-155	[38]	miR-527	[28]	miR-141	[83]	miR-520e	[107]
miR-181b	[39]	miR-550a	[57]	miR-142	[46]	miR-520c-3p	[28]
miR-182	[29]	miR-590-5p	[59]	miR-129-5p	[17]	miR-612	[108]
miR-183	[40]	miR-615-5p	[60]	miR-126-3p	[84]	miR-637	[109]
miR-186	[40]	miR-657	[61]	miR-146a	[32]	miR-1271	[110]
miR-200	[41]	miR-664	[52]	miR-136	[46]	miR-708	[111]
miR-205	[17]	miR-1323	[62]	miR-145	[85]	miR-16-1	[70]
miR-207	[17]			miR-148a	[86]	miR-129	[113]
miR-208-3p	[112]			miR-150	[27]	miR-431	[114]

such as lipid and cholesterol biosynthesis, bilirubin and iron metabolism, oxidative stress-response and hepatic necroinflammation [119].

Although miR-122 expression decreases in HCC, Qi et al. [120] reported elevated levels of serum miR-122 in HCC patients, either in HBV-derived HCC or in HCV-derived HCC patients as compared to patients without HCC.

It is important to underline that miR-122 levels are elevated in inflammation by hepatitis C virus (HCV), because there are four miR-122 binding sites in the HCV genome and miR-122 may promote viral replication [121].

In HBV-related HCC patient, Meng et al. [122] found high serum levels of miR-24-3p.

The serum miR-24-3p were significantly greater in HCC patients than healthy controls and patients with chronic liver disease (CLD) discriminating HCC from CLD patients. Moreover, the combination of serum miR-24-3p and a-fetoprotein (AFP) improves the diagnostic accuracy for HCC prediction compared to each biomarker alone.

Tomimaru et al. described the usefulness of plasma miRNA-21 as a biochemical marker for HCC by comparing the miR-21 expression in patients with HCC and control patients. The authors demonstrated the superiority of the differentiating power of a single measurement of plasma miRNA-21 compared with AFP and, in addition, the combination of plasma miRNA-21 and AFP was significantly stronger than AFP alone [123].

Some previous studies have observed that high levels of serum miR-222 (62), -21, -122, -223 may also be helpful in diagnosis or prognosis of patients with HCC and/or chronic hepatitis [124, 125].

In a retrospective analysis conducted using sera from 105 HCC patients, 107 CLD patients, and 71 normal control subjects, Qu et al. [126] found that serum levels of miR-16 and miR-199a were significantly lower in HCC than in CLD patients or control subjects. Moreover, as a single marker, miR-16 had the highest sensitivity for HCC and the combination of miR-16, AFP, AFP-L3 %, and DCP yielded the optimal combination of sensitivity (92.4 %) and specificity (78.5 %) for HCC.

More recently, Tan et al. in a large cohort of participants (261 HCC patients, 233 cirrhosis patients, and 173 healthy controls) identified and validated 8 serum miRNAs (miR-206, -141-3p, -433-3p, -1228-5p, -199a-5p, -122-5p, -192-5p, and -26a-5p) in order to obtain a miRNA set that provided high diagnostic accuracy for HCC [127]. The authors were also able to differentiate HCC patients from healthy and cirrhosis patients, as compared to previous results obtained from other authors [126].

Wen et al. identified 8 miRNAs (miR-20a-5p, -25-3p, -30a-5p, -92a-3p,-132-3p, -185-5p, -320a, and -324-3p), as well as another 3 miRNAs (miR-192-5p, -21-5p, and -375) in their previous paper [129] as potential markers for early HCC detection [128]. However, after meta-analysis, only 4 miRNAs (miR-20a-5p, miR-320a, miR-324-3p, and miR-375) could be used as preclinical biomarkers for HCC.

Lin et al. [130] in a recent paper assess the performance of circulating miRNAs as biomarkers for early diagnosis in HCC patients. A total of 1416 serum samples were divided in five groups: inactive HBsAg carriers, patients with chronic HBV infection, patients with HBV-related cirrhosis, HCC patients, and healthy controls. Seven miRNAs (miR-29a, -29c, -133a, -143, -145, -192, and -505) were analyzed to detect HCC. This set of miRNAs was validated in two cohorts contained about 500 patients. The seven miRNAs had higher accuracy to identify individuals with HCC from controls as compared to AFP levels. The authors subsequently did a nested case-control study to assess the capacity of this set of miRNAs to detect preclinical HCC in patients with HBV. The results showed that the set of miRNAs detected eight (30 %) cases of HCC at 12 months before diagnosis, whereas AFP20 detected only two cases (7 %). In conclusion the author argues that the selected miRNAs could be valuable to detect preclinical HCC, providing patients with a chance of curative resection and longer survival.

Though the miRNAs evaluated by Lin et al. offers promising diagnostic accuracy and constitutes the proof of concept that miRNA profiling could be an accepted strategy for early diagnosis, Forner believes that further studies are required before its acceptance into surveillance programmes for HCC [131].

Recently, Li et al. have identified circulating miRNAs as novel potential biomarkers for HCC detection by a systematic review and meta-analysis. In the 17 included studies, three circulating miRNAs (miR-21, miR-122, and miR-223) were repeatedly reported three times or more in both HCC patients versus healthy controls and versus other hepatitis or cirrhosis patients. Among them, miR-21 seems to have a highest level of diagnostic efficiency for detection of HCC [132].

Although some miRNAs seem to have greater specificity and sensitivity than traditional diagnostic tests for HCC, additional studies are underway to evaluate the optimal biomarkers.

7.5 Therapies and miRNAs

The treatment of an HCC patient is dependent on several factors, such as stage, size, and grade of the tumor, as well as there are large gaps in our understanding on the molecular mechanisms involved in the pathogenesis of HCC. The elucidation of these diverse mechanisms could help to develop efficient treatment strategies.

In this contest, the miRNAs can be successfully employed to gain further biological and mechanistic understanding of

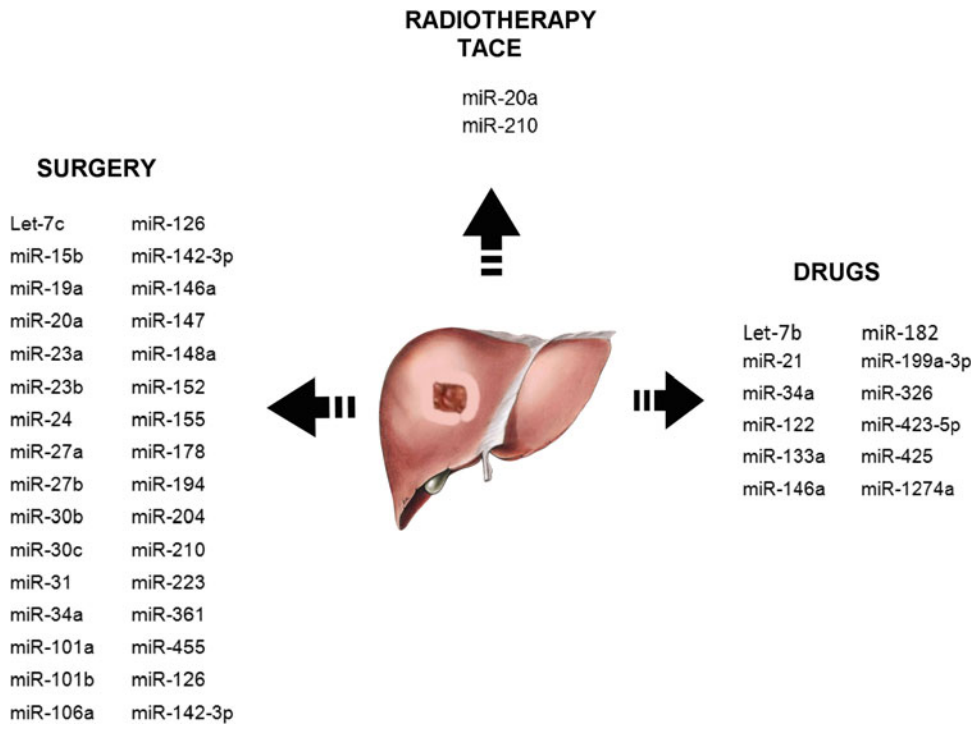

Fig. 7.2 List of miRNAs up- and down-regulated after therapy

metabolism-related HCC, discovering potential biomarkers and identifying novel drug targets.

A number of studies have identified deregulation of miRNA levels during therapy for HCC (Fig. 7.2) and to date, the research has demonstrated that miRNAs are linked to different therapy approaches, such as percutaneous ablation, transcatheter arterial chemoembolization (TACE), conventional chemotherapy, and radiotherapy.

7.5.1 Surgery Therapy

Among different surgery therapies, liver transplantation (LT) remains the best option for patients with HCC even if there is a limited supply of good-quality deceased donor organs. In the allograft transplantation (ALT), the rejection of the organ transplant is a life-threatening complication, while in orthotopic liver transplantation (OLT), the tumor recurrence is main potential cause for the poor outcome. Since there are no established molecular-based standards regarding the preoperative evaluation and selection of HCC patients for LT, Barry et al. [133] have studied the miRNA profile in the formalin-fixed paraffin embedded (FFPE) samples of 40 patients with recurrent HCC within 3 years of transplant and 29 patients without recurrent disease within 3 years to define a miRNA biomarker that reliably distinguishes patients with and without HCC recurrence after liver transplant.

The authors found 67 miRNAs distinguishing patients with and without HCC recurrence after LT. However, they do not propose these miRNAs as biomarkers in clinical adjunct to Milan criteria and suggested further study on this topic.

Han et al. [134] have identified recurrent-related miRNAs in HCC following LT.

The authors suggest a different miRNA expression pattern between HCC samples of patients with recurrence and those with nonrecurrence, proposing six miRNAs (miR-19a, -886-5p, -126, -223, -24, and -147) as biomarkers for prognosis of HCC patients following OLT. Considering Milan criteria, the six miRNAs were also able to predict the patients' OS with 86.7 % of sensitivity and 82.3 % of specificity. Moreover, the metabolic pathway analysis showed important roles of these miRNAs in cell cycle, differentiation, apoptosis, cell migration, angiogenesis and MAPK signaling pathway.

In animal LT model, murine ALT showed miR-146a, -15b, -223, -23a, -27a, -34a, and -451 up-regulated as compared to the expression observed in the syngeneic grafts. In contrast, miR-101a, -101b, and -148a were down-regulated [135].

Outcome after LT is often compromised as a result of various causes such as inadequate graft selection and consequent delayed graft function, recurrence of disease, ischemic cholangiopathy, and usage of immunosuppressive

drugs [136]. Therefore, the need for noninvasive biomarkers to monitor graft quality before, during, and after LT remains.

Farid et al. [137] have analyzed three miRNAs (miR-122, -148a and -194) in serum samples from healthy controls and LT recipients and peri-transplant liver allograft biopsy samples.

The miR-122 and -148a expression in liver tissue was significantly reduced with prolonged graft warm ischemia times, while the serum levels of three miRNAs were elevated in patients with liver injury and positively correlated with aminotransferase levels.

Since the ischemic-type biliary lesions (ITBLs) are the second most common cause of graft loss after LT, Verhoeven et al. [138] have studied miRNA profile in perfusates (graft preservation solutions) and in liver biopsies collected at the end of cold ischemia. The authors demonstrated that cholangiocyte-derived miRNAs (miR-during graft preservation) is predictive of the development of ITBL after LT.

In addition, Lankisch et al. [139] found that the concentrations of miRNA- 517a, -892a, and -106a in bile were increased in patients with ITBLs versus patients with anastomotic strictures or bile duct stones.

Operational tolerance (OT) in liver transplant patients occurs much more frequently than OT of other transplanted organs, however, the rate of OT varies with a range from 5 to 15 % of the LT [140].

The phenomenon of spontaneous immune tolerance without immunosuppressant was first reported by Qian et al. [141] in murine model.

In a recent paper and in an animal model, Wang et al. [142] found that increased levels of murine miR-142-3p, -155 and -152 down-regulated the transcription of IL-6, TGF-Beta-Activated Kinase 1-Binding Protein 2 (TAB 2) and Ca^{2+}/calmodulin-dependent protein kinase (CaMK II) mRNAs involved in tolerance induction.

Another surgery therapy is liver resection. Liver resection is limited to HCC patients with one or two small (5 cm or less) tumors confined to the liver with no invasion of the blood vessels. However, only 5–15 % of HCC patients are currently eligible for this surgical intervention with high rate of metastasis and recurrence. Yang et al. [143] examined the miRNA expression profiling from HCC patients who have had surgical resection to identify recurrence-related miRNAs, using a microarray technique. They found that 32 miRNAs are differentially expressed in tumors of HCC patients with different postoperative survival.

A major obstacle for the treatment for HCC is the high frequency of tumor recurrence even after curative resection and liver transplantation that limits overall survival [144].

Causes of deaths after LT include recurrent HCC in 12 % of patients, recurrent HCV in 4.5 %, whereas 19.5 % of patients died from causes that are to HCV- or HCC-unrelated.

The optimal treatment strategy for patients with recurrent HCC after LT remains unclear, as well as specific biomarkers at the time of surgery that reliably define patients with recurrent HCC after LT have not been identified. Unlike conventional protein-based cancer biomarkers, such as carcinoembryonic antigen (CEA) and a-fetoprotein (AFP), circulating miRNAs packed in microvesicles, called exosomes, possibly have biological and physiological functions in cancer progression, as well as serve as simple biomarkers [145].

Sugimachi et al. found that miR-718 showed significantly different expression in the serum exosomes of HCC cases with recurrence after LT compared with those without recurrence.

Decreased expression of miR-718 was associated with HCC tumor aggressiveness and Homeobox Protein Hox-B8 (HOXB8) seem to be the potential target gene of miR-718, and miR-178 up-regulation was associated with poor prognosis [146].

In acute rejection of LT, Hu et al. [147] found that serum miR-122, -192, and -146a was significantly up-regulated in an animal model, supporting the potential use of miRNAs as noninvasive markers for monitoring graft function since they are noninvasive, stable and easily detected in the blood.

In addition, Wei et al. [148] showed a differential expression of miRNAs in acute rejection between allogeneic and syngeneic solid liver grafts. Of the 226 rats mature miRNAs examined, 46 miRNAs were significantly changed in allogeneic liver grafts as compared to syngeneic liver grafts with increase of miR-204, -210 and -142-3p, and decrease expression of let-7b, -122, -200a and -31. These results demonstrate that miRNAs differentially expressed in liver allografts during acute rejection may play important roles in liver dysfunction post-transplant. Further, the same authors suggest that, the miRNAs, such as miR-142-3p that are expressed in all organ grafts, were associated with lymphocyte alloimmunity during rejection because a major pathological feature of acute rejection after OLT is graft infiltrating lymphocytes (GILs).

Some studies have also proposed the evaluation of the miRNA expression, in association with other methods, such as AST-to-platelet ratio index (APRI), fibroSURE and FibroSCAN, in disease recurrence in the post-liver transplant.

Since hepatitis C infection in immunosuppressed liver transplant recipients is characterized by an accelerated fibrogenesis and faster decompensation of allograft cirrhosis, a set of 9 miRNA signatures associated with progression of fibrosis were differentially expressed and have been

recognized [149]. Three were up-regulated (miR-155, -34a, and -222) and six were down-regulated (miR-23b, -361, -455, -30b, -30c, and -27b).

Joshi et al. [150] analyze liver miRNA expression in carefully matched cohorts of individuals who previously had undergone transplantation for HCV-related liver disease, comparing those with slow versus rapid fibrosis progression, individuals with acute cellular rejection, and control subjects without viral hepatitis. The miRNA expression patterns were seen for all 4 groups.

The miRNA analysis showed increased intra-graft expression of miRNA-146a, -19a, -20a, and let-7e in slow progressors versus fast progressors. In addition, miRNA-19a and miRNA-20a were also specifically detected in the serum of slow progressors. Changes in the miRNA expression regulating fibrogenic, apoptotic, inflammatory, and angiogenic pathways were associated with fast HCV progression. Moreover, the comparison between individuals with rapid fibrosis progression and subjects with acute rejection also revealed different miRNA expression with changes in insulin-like growth factor 1 receptor (IGF-1R) expression and pro-angiogenic pathways associated with vascular endothelial growth factor A (VEGFA) expression, respectively.

Also miRNA expression profiles related to HCV infection and antiviral therapy in adult liver transplant recipients revealed distinct HCV-related miRNA expression, with significant dysregulation of those miRNAs potentially targeting mRNAs of HCV receptors [151].

In conclusion, Amrouche et al. [152] argue that, although the miRNAs still need to be validated in larger patient cohorts, they are not far from being used in transplant clinical practice as usefulness biomarkers. Ongoing multicenter trials should help to further define the clinical utility of miRNA profiles as biomarkers of allograft status and outcome.

7.5.2 Radiotherapy

While the curative treatment for HCC is surgical resection and liver transplantation, most patients are in advanced stage, and lose the chance of surgery and other palliative treatments, such as radiotherapy, transarterial embolization, and chemotherapy, are used.

Since the activation of the PI3 K/Akt pathway is associated with radioresistance, Liu et al. have investigated whether the PI3 K inhibitor, BKM120, can enhance the radiosensitization of HCC cell lines (Huh7 and BNL). The results showed that BKM120 mediated its effect on HCC cells by inhibiting radiation-activated PI3 K/ Akt signals [153].

More recently, Zhang et al. [154] have investigated on the role of miR-20a in HCC cell line subjected to radiation and they showed that miR-20a induced HCC cell radioresistance by activating the PTEN/PI3 K/Akt pathway.

7.5.3 Transcatheter Arterial Chemoembolization (TACE)

Zhan et al. [155] report that serum miR-210 may represent a novel biomarker for predicting efficacy of TACE and overall survival for patients with HCC.

El-Halawany et al. described that in HCC patients treated with TACE using doxorubicin and cisplatin regimen, identified a panel of 12 miRNAs that were significantly deregulated in patients' group of responders compared to nonresponders. Therefore, profiling of these miRNAs in HCC patients prior to treatment may serve as a predictive tool of patients' prognosis [156].

7.5.4 Drug Treatment and miRNAs

Chemotherapy, together with surgery and radiotherapy, has been a main approach for cancer treatment and it has been used as first-line therapy. However, in about 90 % of unsuccessful chemotherapy treatments in advanced cancer patients are present the drug resistance.

The mechanisms of chemotherapeutic drug resistance still remain largely unknown despite extensive investigation and miRNAs could be involved.

Among chemotherapy agents, epirubicin, cisplatin, 5-fluorouracil (5-FU), etoposide, interferon-α (IFN-α), doxorubicin, and their combinations are used in conventional chemotherapy for HCC.

Tomimaru et al. [157] report that the miR-21 in HCC cell lines and clinical HCC samples is a significant modulator of the anti-tumor effect of IFN-α and 5-FU because rend HCC cells sensitive to these drugs.

miR-146a seems to be responsible of the sensitivity of HCC cells to the cytotoxic effects of IFN-α through SMAD4, protein involved in signal transduction of the Transforming growth factor-beta (TGFβ) superfamily [158].

More recently, Ma et al. demonstrated that let-7b binds and represses B-cell lymphoma-extra large (Bcl-xl) mRNA, that is an anti-apoptotic member of the (Bcl-2) family, This phenomenon leads to increased sensitivity of the HCC cells to 5-FU treatment [159].

HCC cells are more resistant to cisplatin, one of the commonly used chemotherapeutic drugs for the HCC treatment, when miR-182 increases during therapy [160].

Also miR 133a and miR 326 contribute to increase the 5-FU and cisplatin sensitivity in HCC cells having as target Bcl-xl, anti-apoptotic protein [161].

Prior to the arrival of sorafenib, doxorubicin was routinely used as a single drug for advanced HCC, but has shown inefficacy, with a response rate of about 15–20 %.

Evaluating the cell resistance to doxorubicin, Fornari's group showed that miR-122, through down-regulation of

cyclin G1, can trigger apoptosis and increase sensitivity of HCC cell lines to doxorubicin [162].

More recently, the same authors report that in HCC cell lines the miR-199a-3p modules the cell cycle and invasion capability by mTOR and c-Met inhibition thus responding to doxorubicin treatment [163].

By sequencing analysis, Zhang et al. found a total of 269 known miRNAs significantly differentially expressed, of which 23 were up-regulated and 246 were down-regulated, in HCC cell lines treated with doxorubicin, indicating that part of these miRNAs might be involved in the development of doxorubicin resistance [164].

Since HCC is frequently resistant to conventional chemotherapy, clinical development of novel therapeutic agents against HCC has begun in earnest. Thus far, a series of adjuvant therapies for HCC have emerged, including small molecular target agents, monocolonal antibodies, multikinase inhibithors. Some agents such as sorafenib have shown an advantage in prolonging the overall survival time, and has been approved by Food and Drug Administration for the treatment of advanced HCC.

Using miRNA microarray, Zhou et al. found that sorafenib alter the expression of 14 miRNAs in HCC cells and among them, miR-1274a could be significantly up-regulated after adding sorafenib and greatly reduce ADAM metallopeptidase domain 9 (ADAM9) expression. ADAM9, present in HCC tissue, claves heparin-binding epidermal growth factor [165].

Bai et al. [166] demonstrated that miR-122, abundantly expressed in hepatocytes but barely detectable in human HCC cells, inhibited tumorigenic properties of miR-122-expressing HCC cells and sensitized these cells to sorafenib.

Patients with lower miR-34a expression had significantly poorer overall survival because miR-34a represses the Bcl-2 mRNA translation, an anti-apoptotic factor. The restoration of miR-34a reduced cell viability, promoted cell apoptosis and potentiated sorafenib-induced apoptosis [167].

On the contrary, up-regulation of miR-182 increases sorafenib resistance and enhances HCC tumorigenicity [168].

Secretory miR-423-5p was up-regulated in patients treated with sorafenib promoting autophagy. The increase of this miRNA was also correlated to positive response to therapy in 75 % of patients that showed partial remission or stable disease after 6 months from the beginning of therapy [169].

Therefore, miR-423-5p could predict the response to sorafenib therapy in patients with HCC [170].

Moreover, it is possible to evaluate miRNA expression patterns from HCC tissue biopsies as potential biomarkers in patients under sorafenib treatment [171].

Today, the current therapies for HCC are challenged and new molecular therapies for HCC, including erlotinib, cetuximab, bevacizumab, and sunitinib, have been tested in clinical trials for HCC. In addition, primary and/or acquired resistance in the tumor could be overcome by novel combinational therapies. RNA interference-mediated gene inactivation, alone or in combination with other current therapies, provides novel promising therapeutics that can improve cure rate and overcome resistance mechanisms to conventional therapeutics.

The expression of miR-146a is down-regulated in HCC tissues compared to the adjacent noncancerous hepatic tissues and Huang et al. [172] explore the effect of miR-146a mimic, similar endogenous mature miR-146a, with and without cetuximab in an in vitro model. The miR-146a mimic alone decelerated the cell growth in all HCC cell lines tested, but when it was combined with cetuximab, a strongest effect was obtained (synergistic effect). Therefore, the authors conclude that the application of miR-146a mimic might thus be a promising approach to HCC therapies in the future [173].

To investigate whether the cooperative activity of erlotinib and miR-34a has utility in HCC, Zhao et al. [174] probed this combination in cell models of HCC. Liver cancer was chosen as test platform because erlotinib is moderately effective in patients with advanced liver tumor as a single agent and failed to prolong overall survival and time-to-progression in combination with sorafenib. Moreover, miR-34 levels are low or undetectable in liver cancer cells. Data showed a strong synergy between erlotinib and miR-34a mimic (also called MRX34) in all HCC cell lines tested and an enhanced efficacy with erlotinib-miR-34a combination where erlotinib alone was insufficient.

Moreover, since MRX34, a liposomal nanoparticle loaded with synthetic miR-34a mimics, has recently a phase 1 clinical trial [175], clinical testing of the erlotinib-miR-34a combination could be quickly initiated.

7.5.5 miRNAmimics and Anti-miRNAs as Drugs

Generally, the therapeutic application of miRNAs involves two strategies. These include inhibition strategy and replacement strategy.

miRNA replacement, involves the reintroduction of a tumor suppressor miRNA mimic to restore a loss-of-function. However, to date, few tumor suppressor miRNAs have been discovered for which the proof of concept of miRNA replacement therapy has been demonstrated in preclinical animal models of cancer.

The inhibitory approach is more commonly accepted and conceptually follows rules that also apply to small molecule inhibitors and short interfering RNAs (siRNAs). These miRNA antagonists are oligonucleotides with sequences complementary to the endogenous miRNA (called

anti-miRNA antisense oligomer or AMO). The aim is to inhibit oncogenic miRNAs, generally increased in tumor tissues, using these miRNA antagonists [176, 177].

Among the various forms of AMOs, antagomiR that has its end conjugated with a cholesterol moiety has demonstrated most impressive effectiveness against target miRNAs, intracellular stability, particularly for in vivo applications [178].

However, modification to stabilize AMOs to nuclease degradation and improve affinity for target miRNAs are necessary for their miRNA-antagonizing activities in cell culture and animals.

There have been several generations of AMO designs. The first generation utilized 2′-*O*-Methyl RNA nucleotides with phosphorothioate internucleotide linkages positioned at both ends of AMO to prevent exonuclease attack. Another chemical manipulation is 2′-sugar-AMOs. A recent study discovered a compound, *N*,*N*-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine (ZEN), that blocked exonuclease degradation and created a new generation called ZEN-AMO with an improved effectiveness [179].

Delivery of AMOs requires in vitro transfection or in vivo uptake into target cells and this appears to be one of the most important factors influencing the activity of the miRNA.

At present, there are difficulties with conventional methods of transfection that result in low delivery efficiency. In order to increase the effectiveness of AMO delivery a functionalized gold nanoparticles was proposed. The gold nanoparticles increase delivery efficiency by conjugating with a cargo DNA that anneals to the AMO using complementarity. Another in vivo method for delivery supported by results in mice is the injection of AMOs intravenously or intratumoral tissue, as well as viral vector-based delivery or exosomes [177].

In a HCC mouse model, strategies for miRNA replacement therapies have been developed using miR-26a, miR-122, and miR-124 [79, 180, 181].

In contrast, inhibition of miR-221 lengthened survival, reduced the nodule number and retarded tumor development [182].

Using mouse Myc-induced liver tumors, Kota et al. identified that compared with other miRNAs, miR-26a demonstrated the most notable change in expression. Furthermore, the restoration of miR-26a expression using an adenoviral vector (AAV) delivery system in the same model inhibited proliferation and promoted cancer cell apoptosis, but did not induce apoptosis in the nonmalignant hepatocytes [183].

As inhibition strategy, a study by Park et al. [184] revealed that the intravenous administration of a cholesterol-modified isoform of anti-miR-221 oligonucleotide, into an orthotopic mouse model of liver cancer, reduced tumor cell proliferation and increased markers of apoptosis.

This suggested that the targeted inhibition of miRNA contributed to the successful treatment of HCC.

An in vitro study by Ma et al. revealed that the up-regulated expression of miR-122 in metastatic Mahlavu and SK-HEP cells inhibited intrahepatic metastasis, and led to a decreased rate of tumorigenesis and angiogenesis [185].

Therefore, the development of miRNA-based therapies could be of potential value for future HCC treatment regimens.

The biological function of miR-99a deregulation in HCC remains unknown and Li et al. [75] inhibited significantly tumor growth and reduced the AFP level in hepatocellular carcinoma-bearing nude mice by intratumoral injection of cholesterol-conjugated miR-99a mimics.

On the contrary, miRNA-21 has been shown to be up-regulated in HCC and Wagenaar et al. have developed potent and specific single-stranded oligonucleotide inhibitors of miR-21 (anti-miRNAs) and used them to interrogate dependency on miR-21 in a panel of liver cancer cell lines. Robust induction of caspase activity, apoptosis, and necrosis was noted in anti-miR-21-treated HCC cells. Furthermore, ablation of miR-21 activity resulted in inhibition of HCC cell migration and suppression of clonogenic growth [186].

In conclusion, the miRNA revolution has provided the industry with multiple new opportunities for the identification of new drug targets. These revelations, coupled with recent advances in anti-miR chemistries, suggest that the regulation of miRNAs may be the next innovation in pharmaceutical research. [187]

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Nontumor Prognostic Factors in Hepatocellular Carcinoma

8

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Contents

8.1 Introduction

Liver cancer, mainly hepatocellular carcinoma (HCC), is the second leading cause of cancer death worldwide, and its prognosis is still dismal (5-year overall survival rate generally below 15 %) (GLOBOCAN 2012, globocan.iarc.fr). In the United States, the incidence of HCC has significantly increased over the past 30 years and it is currently the fastest rising cause of cancer-related mortality [1]. In parallel, the advent of high-throughput molecular technologies in biomedicine has opened new paths in oncology research. For instance, the measurement of biomarkers, defined as an objectively measured characteristic that describes a normal or abnormal biological state in an organism [2], may allow to measure the risk of developing cancer in a specific tissue or, alternatively, may measure risk of cancer progression or potential response to therapy. A framework for the development of biomarkers has been described from biomarker discovery to their validation and clinical implementation [3]. In recent years, such efforts have been encouraged, for example, by the precision medicine initiative, that includes an investment of $70 million to the National Cancer Institute (NCI), to "scale up efforts to identify genomic drivers in cancer and apply that knowledge in the development of more effective approaches to cancer treatment" [4].

However, these novel approaches come with new challenges, such as high biostatistical/computational requirements, and the need for novel validation strategies due to the high number of candidates generated. One author has aptly summarized these novel challenges by coining the expression "$1000 genomic test [but] $100,000 genomic analysis" emphasizing the computational challenges at analyzing and making sense of such high level data [5]. Despite an increased understanding of the carcinogenic steps leading to the HCC development, there remains a significant dearth in characterizing non-HCC clinical factors associated with HCC onset and prognosis [6]. Herein, we will provide a broad overview of non-HCC clinical and molecular factors associated with its development and outcome.

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8.2 Factors Associated with the Development of a First HCC

Even in subjects with full-blown cirrhosis, the risk of HCC development varies widely from 2.4 % in 7 years to 21 % in 5 years depending on different factors including etiology of liver disease [7]. Clinical practice guidelines [8, 9] recommend HCC surveillance with abdominal ultrasound every 6 months in at risk populations. Recent studies further suggest how surveillance may improve HCC outcomes [10]. However, implementation is still a major issue with only 12 % of hepatitis C virus (HCV) cirrhotics having routine surveillance in one US Veterans Affairs series, and only 2 % of HCV patients who developed HCC had previous appropriate surveillance [11, 12]. In addition to measures aimed at improving implementation of surveillance programs, the development of additional risk biomarkers, less operator-dependent, could potentially increase success rates of surveillance in certain areas.

8.2.1 Clinical Prognostic Factors

A number of clinical-based systems have been proposed to assess risk of HCC development in the setting of liver disease (Table 8.1). Most of them include variables related to the underlying liver dysfunction and/or the degree of portal hypertension. More recently, two factors have emerged as significantly associated with hepatocarcinogenesis: obesity and insulin resistance. There is increasing evidence of an association between obesity and multiple types of cancer, including liver cancer. For instance, in a large cohort of more than 900,000 US adults, body mass index (BMI) was associated with death from multiple cancers and a BMI over 40 kg/m^2 led to an increase of cancer death rates of 52–62 % [13]. As a result, the American Society of Clinical Oncology stated that "Obesity is a major under-recognized contributor to the nation's cancer toll and is quickly overtaking tobacco as the leading preventable cause of cancer" [14]. In this study, liver cancer was associated with the highest increase in cancer

Table 8.1 Examples of clinical risk scores associated with development of HCC

Risk score	Etiology of liver disease	Cirrhosis (%)	Variables	Reference
ADRESS-HCC	HCV (46 %) Alcohol (18 %) NASH (18 %) HBV (3 %)	100 %	Age, diabetes, race, etiology of cirrhosis, sex, and severity of liver dysfunction (Child-Pugh score)	[78]
Velazquez et al.	Alcohol (59 %) HCV (29 %) HBV (7.5 %)	100 %	Age, anti-HCV positive, prothrombin time and platelet count	[79]
UM regression model	HCV (47 %) Cryptogenic (19 %) Alcohol (15 %)	100 %	AFP and gender	[80]
GAG-HCC	HBV	15 %	Age, gender, HBV DNA, core promoter mutations, cirrhosis	[81]
CU-HCC	HBV	38 %	Age, albumin, bilirubin, HBV DNA, and cirrhosis	[82]
LSM-HCC	HBV	31 %	Liver stiffness, age, albumin, HBV DNA	[83]
REACH-B	HBV	0 % discovery cohort, 18 % validation cohort	Sex, age, ALT, HBeAg status, and serum HBV DNA level	[23]
Risk index	HCV after SVR	10 %	Age, AST, platelet count	[29]
score$_{HCC}$	HCV after SVR	30 %	Age, AFP level, low platelets and advanced fibrosis	[27]
Chang et al.	HCV after therapy	45 % fibrosis stage 3–4	Age, male sex, AFP level, low platelet, advanced fibrosis, HCV genotype 1b, and non SVR	[84]
El-Serag et al.	HCV	100 %	AFP, ALT, platelets, interaction terms, and age	[85]
HALT-C model	HCV	41 %	Age, race, alkaline phosphatase, esophageal varices, ever smoked, and platelet count	[25]
REVEAL-HCV	HCV	4 %	Age, ALT, AST/ALT ratio, HCV RNA, cirrhosis, and HCV genotype	[86]
Liver stiffness measurement	HBV	50 %	Liver stiffness measurement	[87]
FIB-4	HBV	10 %	FIB-4 (AST, ALT, platelets, age)	[88]

BCLC, Barcelona clinic liver cancer; CLIP, Cancer of the liver Italian program; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; JIS, Japan Integrated Staging score; MELD, model for end-stage liver disease; MELD-Na, model for end-stage liver disease-sodium, SLICER, Singapore Liver Cancer Recurrence; UM, University of Michigan

death in men according to BMI [13]. A systematic review of 11 cohort studies observed that the risk of developing HCC was 17 % higher in overweight and 89 % higher in obese individuals. It remained unclear whether this was mediated through nonalcoholic fatty liver disease (NAFLD) or through other obesity-specific mechanisms such as activation of pro-oncogenic pathways in a context of low-grade inflammation [15]. Insulin resistance and type 2 diabetes (T2D) have also been associated with cancer development, in particular HCC. For instance, in a systematic review including 13 cohort studies, diabetes was associated with the development of HCC (pooled risk ratio 2.5) independent of alcohol use or viral hepatitis [16]. In another study, diabetes was associated with a 2–3 fold increase in HCC risk, regardless of the presence of other major risk factors although the effect of NAFLD was not adequately addressed in this study [17]. A proposed mechanistic link includes insulin resistance and hyperinsulinemia leading to increased levels of Insulin-like growth factor 1 and subsequent signaling inducing cellular proliferation and apoptosis inhibition [18]. Interestingly, the association between diabetes and HCC has been called into question by a recent systematic review of all published meta-analyses, where T2D was linked to intrahepatic cholangiocarcinoma and three other cancers but not HCC. Authors used stringent quality criteria for study selection, allowing the inclusion of only 6/27 (22 %) meta-analyses and potentially limiting the generalization of their findings [19].

The degree of liver fibrosis has also been repeatedly associated with the development of HCC across all etiologies of liver disease. HCC development in cirrhosis is a decade long stepwise process in the setting of an inflammatory, fibrogenic, and carcinogenic tissue microenvironment in the liver [20]. The occurrence of HCC in the absence of cirrhosis is well described in hepatitis B virus (HBV) due to direct oncogenic viral mechanisms including DNA integration of HBV components. However, in recent years, a growing number of reports have underlined that HCC may also occur without cirrhosis in patients with NAFLD. For instance a recent large cohort study including 1500 Veterans with HCC confirmed that patients with NAFLD or metabolic syndrome had more than five times the risk of developing HCC in the absence of cirrhosis compared with HCV-related HCC [21]. This is even more worrisome in the context of the rapidly increasing NAFLD/obesity epidemic. A recent estimate suggests that NAFLD represents the third most prevalent etiology of HCC in the United States [22]. With a 9 % annual increase in NAFLD-related HCC cases in the US, it is likely that NAFLD may shortly overtake HCV and alcohol as the main etiologic factors for HCC development [22].

Multiple other clinical factors have been found to be associated with the development of HCC and have been integrated into a wide range of clinical scores, mostly in the context of hepatitis-related HCC (Table 8.1). In patients with HBV, age, viral parameters (e.g., high HBV DNA, core promoter mutations, HBeAg status), degree of liver dysfunction and inflammation have been consistently shown to be associated with risk of HCC development (Table 8.1). A study enrolling 3584 HBV Asian subjects without cirrhosis identified sex, age, ALT, HBeAg status, and serum HBV DNA levels as factors associated with HCC development, and developed a 17-point risk score predicting HCC risk at 3, 5, and 10 years with a 5-year probability of developing HCC ranging from 0 to 47 % [23]. However most of these studies were derived and validated in Asian populations, where the predominant HBV genotypes are B and C. These risk scores have been shown to underperform in Caucasians, particularly in subjects treated with antivirals [24]. These findings underscore the need for better tools to refine HCC risk prediction, particularly in subjects receiving antiviral therapy. In HCV patients, variables, such as age, portal hypertension, and presence of cirrhosis and ALT levels have all been shown to be significantly associated with HCC risk (Table 8.1). Results from the large Hepatitis C Antiviral Long-Term Treatment Against Cirrhosis (HALT-C) trial that enrolled 1005 subjects reported age, race, platelet count, serum alkaline phosphatase, esophageal varices, and smoking as significantly associated with HCC development [25]. The impact of direct-acting antiviral (DAA) in the epidemiology of HCV-related HCC is still to be determined, but it will likely significantly reduce the burden of HCV-related HCC in Western populations. A controversial area is the risk of HCC development in HCV patients that cleared the virus. Different attempts have failed to identify clear risk factors in these patients [26–30] although one proposal, the $score_{HCC}$, found that old age, high α-fetoprotein (AFP), low platelet counts and fibrotic stage was associated with development of HCC [27]. In addition, whether the absolute and relative reduction of HCC risk seen after interferon-based therapy for HCC will be reproduced after SVR post non-interferon-based therapy is still unknown.

Although numerous clinical factors have been shown to be associated with risk of development of HCC, especially in HCV and HBV-related HCC, the rapidly shifting epidemiological landscape of HCC development has underlined the important limitations inherent to this approach. As discussed below, there is a hope that assessing molecular-based biomarkers will allow cross-etiology improved risk stratification and ultimately better selection of high-risk patients for chemoprevention and surveillance trials.

8.2.2 Molecular Prognostic Factors

A biomarker is an objectively measured characteristic that describes a normal or abnormal biological state in an organism by analyzing biomolecules, such as DNA, RNA,

protein, peptide, or biomolecule chemical modifications [2, 31]. More specifically in terms of clinical utility, a cancer biomarker may measure the risk of developing cancer in a specific tissue or, alternatively, may measure risk of cancer progression or potential response to therapy. Theoretically, any type of biomolecule can be used as a biomarker although advantages and limitations are associated with each type of biomarker. DNA structural alterations such as somatic gene mutations are relatively easy to test because of stability of DNA molecule and the nature of the measurement; however, assessment of the consequence of each individual mutation remains challenging. RNA expression profiling has been most extensively investigated to identify signatures able to capture the biological state of a given specimen [32, 33]. However, measures can be largely affected by experimental variation such as differences in assay protocol and platform. Although, gene-expression signatures of cancer risk development are not yet currently available in the clinic, there are a few examples of gene-expression signature prognostic tests for breast cancer recurrence, such as Mammaprint, based on the measurement of 70 genes to predict breast cancer recurrence after chemotherapy [34] or the Oncotype Dx Breast Cancer Assay measuring 21 genes predicting breast cancer recurrence in women with invasive breast cancer [35, 36]. The 21-gene expression assay for breast cancer was recently validated in a large prospective trial of more than 10,000 women with breast cancer identifying a favorable risk subgroup of patients with very low 5-year recurrence rates with endocrine therapy alone [37]. Similar tests are also available for colon and prostate cancer, all of which analyze gene expression in tumor tissue [38, 39]. In fact, overlap of signature genes defined in independent datasets is very small even if the signatures are identified from a similar study design [33]. Despite the low concordance of signature gene membership, one interesting property of gene-expression signatures is their ability to capture similar biological characteristics across patient cohorts, assay technologies, or even species [40]. In fact, poor prognosis predictions made by multiple independent signatures in HCC generally overlapped despite distinct selected genes [41]. Similarly, in breast cancer, one report found that four out of five gene expression prognostic profiles showed significant agreement in outcome prediction despite little overlap in gene identity [42].

A number of molecular biomarkers have been developed predicting the risk of a first HCC in subjects with liver disease (Table 8.2). A 186-gene expression signature, derived from nontumoral liver tissues of subjects undergoing hepatic resection for HCC, has proven prognostic not only for HCC recurrence but also for liver disease progression, HCC development and overall survival in subjects with early stage HCV cirrhosis [43–45]. The signature was present in the liver of rodent models of fibrosis/cirrhosis-driven HCC, and the poor prognosis pattern of the signature was reversed in association with the HCC chemopreventive effect of an FDA-approved EGFR inhibitor, erlotinib [46], which is now being tested in a phase one trial with the gene signature as a companion biomarker (ClinicalTrials.gov, NCT02273362). Similarly, liver tissue-derived transcriptome signatures have been associated with multicentric HCC development and late recurrence after curative HCC treatment attributable to de novo HCC development [47–49]. A prognostic index including a 122-gene stellate cell gene signature, derived by comparing multiple tissue transcriptomic profiles was associated with multiple clinical outcomes, including development of HCC and liver disease progression [50].

Several germline single nucleotide polymorphisms (SNP) were reported to be associated with increased HCC risk and other liver disease-related outcomes (Table 8.2) although few of them are replicated in independent patient series/cohorts [51]. One of the most studied SNP associated with HCC in this setting is a variant in patatin-like phospholipase domain-containing 3 (PNPLA3). A number of reports have underlined that the *PNPLA3* I148 M variant leads to an accumulation of lipids in hepatocytes through increased triglyceride lipogenesis and impaired hydrolysis [52]. One systematic review of individual data of European patients showed that a variant (rs738409 C > G, encoding for I148 M) in the *PNPLA3* gene was strongly associated with the development of HCC, in particular in the setting of alcoholic liver disease although the association was also significant for HCV-related HCC [53]. Another systematic review confirmed the known association of *PNPLA3* polymorphism with fibrosis severity but also identified an association with an increased risk of HCC although the association was restricted to NAFLD or alcohol-related cirrhosis in subgroup analysis and not other etiologies of cirrhosis [54]. Another SNP, the *EGF* 61*G allele was associated with HCC risk in a prospective cohort of patients with HCV-related advanced fibrosis (39 % cirrhotic) [55, 56]. Despite diverse allele frequency across patient populations, association between the *EGF* genotype and HCC risk remains significant and independent of patient race [57]. Interestingly, EGF ranks among the top up-regulated genes in the 186-gene signature associated with HCC risk in early stage HCV-cirrhosis [43, 45]. Multiple genome wide association studies (GWAS) have identified other SNPs associated with HCC risk, in particular in the context of viral hepatitis. One intronic SNP in the *DEPDC5* locus was associated with HCC risk in HCV-infected patients even after adjustment for other risk factors [58]. The key role of the immune response in the development of HCC was underlined by the finding that three susceptibility loci within the class II MHC complex were associated with HCC in hepatitis B and C Asian patients, as well as a variant within a *PTEN* homolog *TPTE2* [59]. Yet another GWAS in hepatitis B identified variants in *KIF1B*, *UBE4B,* and *PGD* to be

Table 8.2 Non-HCC molecular parameters associated with HCC development and tumor recurrence

Tissue	Method	Platform	Risk score	Number of patients	Dominant etiology	Outcomes	Type of samples	Cirrhosis rate (%)	Reference
Non-tumoral liver tissue	Gene expression	DNA microarray assay (Illumina)	186-gene signature	216	HCV	Overall death, progression to advanced cirrhosis, HCC	FFPE liver needle biopsy	100	[43–45]
		DNA microarray assay (Illumina)	223-gene signature (HIR signature) 65-gene signature	396	HBV (89 %)	Late recurrence (223-gene signature), early recurrence (65-gene signature)	Frozen hepatic tissue	78	[48]
		ABI PRISM 7700	Immune response signature	115	HBV (96 %)	Recurrence, poor survival	Frozen hepatic tissue	91	[66]
		cDNA microarray (Agilent Human 1)	36-gene signature	40	HCV	Multicentric occurrence	Frozen hepatic tissue	43	[47]
		cDNA microarray (Affymetrix U133A array)	Activated HSC signature	319	HBV (92 %)	HCC recurrence and survival	Frozen hepatic tissue	87	[72]
		DNA microarray assay (Illumina)	HSC signature	82	HCV	Overall survival	FFPE liver needle biopsy	100	[50]
	miRNA profiling	miRBase	56 miRs	73	HCV	Late recurrence	Frozen hepatic tissue	51	[71]
Peripheral blood	SNP		*EGF*	816	HCV	6-year HCC risk	Blood	39	[56]
			PNPLA 3	532	Alcohol (52 %), HCV (48 %)	6-year HCC risk	Blood	100	[89]
			MPO	205	HCV	HCC risk	Blood	100	[62]
			CAT	205	HCV	HCC risk	Blood	100	[62]
			HFE	301	Alcohol (54 %), HCV (46 %)	HCC risk	Blood	100	[64]
			DEPDC5	212	HCV	HCC risk	Blood	NA	[58]
			TPTE2 DDX18	386	HBV, HCV	HCC risk	Blood	NA	[59]
			KIF1B	355	HBV	HCC risk	Blood	NA	[60]
			MICA	721	HCV	HCC risk	Blood	NA	[61]

identified with HBV-induced HCC [60] whereas another report found that a risk allele in the *MICA* gene was associated with progression to HCC in HCV-infected subjects [61]. A SNP in an antioxidant enzyme (i.e., *MPO)* was associated with HCC risk in a prospective study in HCV-cirrhosis [62]. Excess iron deposition in hepatocytes may be a consequence of genetic causes, such as C282Y *HFE* mutation leading to genetic hemochromatosis, or acquired causes such as viral hepatitis or excess alcohol use [63]. The association between liver iron overload and ALD-HCC risk was well characterized in a cohort of 301 cirrhotic subjects including 162 subjects with ALD [64]. In this study, hepatic iron overload, as assessed by hepatic histology and C282Y HFE mutation were both associated with the development of HCC in ALD

but not HCV-related liver disease underlining the importance of this risk factor, especially in populations with a high prevalence of *HFE* mutations.

8.3 Molecular Markers of HCC Progression

Although clinical-based scores have allowed stratifying HCC patients based on their recurrence risk, there is compelling data suggesting that molecular-based tools may accurately capture the complexity of the disease and help predict its outcome. Indeed, gene-signatures—using high-throughput technologies and hence, interrogating comprehensive panels of genes-are able to predict HCC recurrence along with other clinical variables [41]. Most prognostic gene signatures reported so far were generated using so-called "convenience samples" from surgically resected patients [65]. This provides the lowest level of evidence in biomarker studies [3]. Also, the fact that specific radiological features allow for a confident HCC diagnosis results in a significant reduction of tissue availability for biomarker studies. This is particularly problematic in patients at more advanced stages, where molecular targeted therapies are recommended, and in whom gene-signatures may be of particular relevance for stratification purposes. These are limitations that could negatively impact the implementation of gene expression signatures in clinical practice, and somehow justifies the relative low amount of studies exploring nontumoral molecular markers of progression.

8.3.1 Signatures Derived from Adjacent Nontumoral Liver

As previously discussed, a 186-gene signature generated from nontumoral liver tissue of patients undergoing curative resection for HCC highly correlated with survival [45], but also with progression to cirrhosis and to HCC occurrence in patients with HCV [43, 44]. Analysis of the HCC environment also identified gene-signatures predicting tumor dissemination and survival, underlying a Th1/Th2 inflammatory signal shift [66]. It is then likely that genomic information coded in the adjacent nontumoral tissue may contribute to both, early and late recurrences. As depicted in Fig. 8.1, there are two patterns of recurrence after liver resection: early recurrence (<2 years), also known as "true metastasis" and late recurrence ($\geq$2 years), mainly de novo tumors [67]. While signatures derived from tumoral tissue were initially able to predict true metastasis [68, 69], signatures capturing de novo HCC development were mostly derived from nontumoral tissue [48, 70, 71]. In addition to the 186-signature, a recent multicentric study including 396 HCC patients identified a 233-gene signature significantly associated with late recurrence [48]. In addition to mRNA, there are a number of studies using miRNA profiling as a source for biomarker discovery. Consistent with gene expression, miRNAs from the tumor tended to better predict early recurrence, whereas those from the adjacent tissue more accurately predicted de novo carcinogenesis [71]. Similarly, a specific gene-expression signature in the surrounding liver was capable of reflecting the risk of multicentric HCC [47], further suggesting its involvement in identifying a favorable microenvironment for metastatic tumor spread. Since activated hepatic stellate cells (A-HSCs) are key players in the pathogenesis of liver fibrosis, their contribution in HCC occurrence has been recurrently suggested. Two genomic studies have recently addressed this issue reaching similar conclusions. One study developed a 122-gene signature, specific to HSC, that when tested in different cohorts of patients was able to predict risk of HCC development and patient's survival [50]. Similarly, another report found a

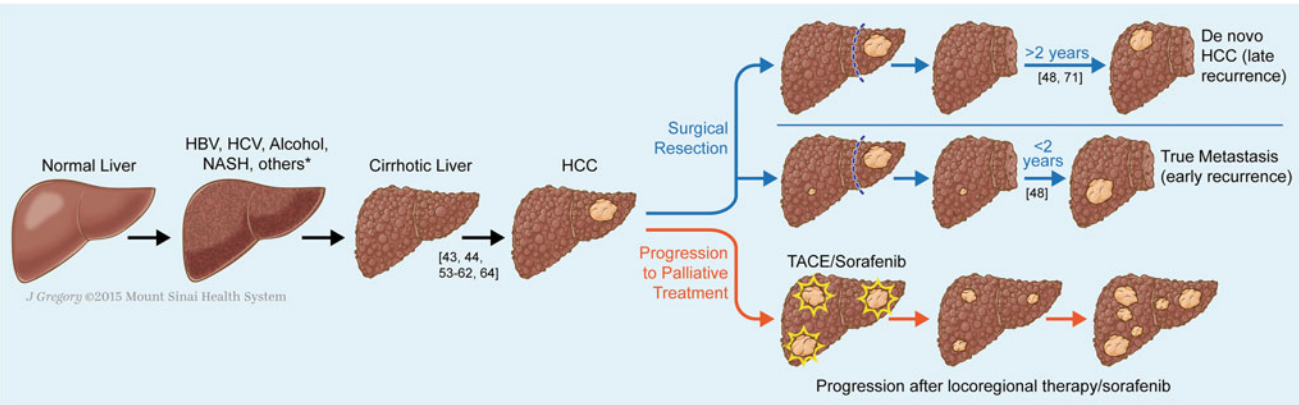

Fig. 8.1 Overview of non-HCC prognostic factors in the context of the natural history of HCC. A number of clinical (Table 8.1) and non-tumoral molecular markers (Table 8.2) have been shown to be associated with the development of HCC across a wide variety of etiologies. Once HCC develops, distinct clinical and molecular markers are associated with recurrence following surgical resection (*blue arrows*) or progression after palliative treatment options such as transarterial chemoembolization or sorafenib (*red arrows*). Reference numbers of selected molecular risk factors linked to progression are highlighted

37-gene signature specifically reflecting A-HSCs and associated with postoperative recurrence and survival [72].

Minimally invasive approaches are currently under development aiming at overcoming the need for tissue biopsies to generate molecular-based predictors. The molecular analysis of tumor's byproducts released into body fluids, namely "liquid biopsy" may offer a wide range of opportunities to interrogate the genomic profile of tumors, via a simple blood test [73]. In addition, noninvasiveness of radiological imaging techniques may also offer similar advantages. Specific radiological features of the tumor may be regarded as surrogate markers of gene-expression signatures. A study showed how the combination of 28 computed tomography (CT) traits could reproduce 78 % of the global gene-expression profile in HCC samples [74]. Similarly, a recent study identified a subgroup of HCC whose radiological characteristics on magnetic resonance imaging correlated with differentiation level and gene-expression [75].

8.4 Conclusion

Clinical parameters alone are probably still insufficient to accurately stratify patients based on their risk for HCC development or recurrence/progression following conventional treatments. In terms of early HCC detection, adequate implementation of surveillance programs with abdominal US is still a major issue, despite being recommended in clinical practice guidelines [76]. Additional challenges lie ahead with the shifting etiological landscape of liver diseases and the advent of novel direct-acting antivirals against the hepatitis C virus (HCV) leading to over 90 % sustained virological response (SVR) rates [77]. However, it has become clear that subjects with SVR are still at risk of developing HCC, albeit at a lower rate. Risk factors in this population are still under scrutiny [28]. In addition, the current surge in NAFLD incidence has also uncovered a potential risk of HCC arising on the background of non-cirrhotic liver although definitive estimates are still lacking [18]. Facing these significant unmet needs, molecular tools such as gene signatures could improve currently available prognostic models. Hopefully, these new approaches will contribute to improve outcomes of HCC patients by improving selection methods, better allocation of resources and enable tailored medical interventions.

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Gut Microbiota and HCC

9

Sven H. Loosen, Christoph Roderburg, and Tom Luedde

Contents

9.1 Gut Microbiota

The term microbiota, mainly coined by Nobel prize-winning molecular biologist Joshua Lederberg, describes "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" [1]. The human gut microbiota consists of about 100 trillion microbial cells including bacteria, viruses, protozoa and other organisms. The total number of genes aggregated in these cells is believed to surpass the number of human genes by the factor of 100 [2, 3]. Strict anaerobes represent the majority of gut microbiota with *Bacteroidetes* and *Firmicutes* being the most prevalent phyla [4]. The localization of the intestinal microbiota is not homogenous. Starting with 10^1 cells/g in the stomach, the number and diversity of microbial cells increases up to a maximum of 10^{12} cells/g in the colon [3]. Microbiota perform a wide range of vital functions essential to health maintenance, including metabolic functions such as food processing or synthesis of vitamins as well as structural functions. Furthermore, it secretes a number of biologically active metabolites with various functions like the metabolism of toxic compounds or the inhibition of pathogens via the secretion of IgA or antimicrobial factors [5].

9.2 Gut Microbiota in Liver Disease

From a historical viewpoint, the connection between microbiota and the development of diseases dates back to ancient Egypt when physicians presumed that putrefaction of the stools associated with an absorption into the general circulation leads to fever and the formation of pus [6]. In the nineteenth and early twentieth century, biomedical and bacteriologic studies suggested that the degradation of protein in the colon by anaerobic bacteria generates toxic amines. Metchnikoff, one of the foremost proponents of autointoxication hypothesized that intestinal toxins may shorten life span and Sir Arbuthnot Lane even suggested colectomy as the only "cure" for mental health disorders related with

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autointoxication and focal infections [7, 8]. The first firm scientific evidence for a role of gut microbiota in liver disease was published in the 1950s when Philips et al. were able to show a linkage between the absorption of nitrogenous substances from the intestine and the development of hepatic coma in patients suffering from liver cirrhosis [9]. These findings were followed by the analysis of the bacterial content of the small intestine in normal and cirrhotic patients and the first attempt of using antibiotic treatment as a therapy for hepatic encephalopathy in the 1950s [10, 11]. From today's point of view, many of the underlying disease-causing connections between gut microbiota and the development of hepatic diseases are still not fully understood but various scientific findings from the last decades have led to the establishment of different explanatory approaches.

It has been shown that chronic liver disease is associated with an augmented translocation of intestinal bacteria which leads to increased bacterial infections such as spontaneous bacterial peritonitis (SBP), urinary tract infections, and pneumonia [12, 13]. Bacterial translocation to mesenteric lymph nodes was observed in rodents as well as patients with advanced liver cirrhosis [12, 14, 15]. However, it seems that the translocation of bacterial components, termed pathogen-associated molecular patterns (PAMPs), is even more important than translocation of viable microorganisms. The liver receives about 70 % of its blood supply from the gut through the portal vein system and is thereby decisively affected by translocated PAMPs such as lipopolysaccharide (LPS) from the intestinal microflora. Seki et al. demonstrated a crucial role for gut-driven portal LPS as a requirement for developing liver fibrosis during chronic liver disease [16]. Antibiotic treatment of mice resulted in a reduced increase of plasma LPS after bile duct ligation and histological analysis of liver samples showed a reduced infiltration of macrophages. Consequently, these animals were protected from developing liver fibrosis and cirrhosis, indicating a substantial role of intestinally derived LPS in hepatic disease [16]. These findings were corroborated by observations made by Gómez-Hurtado and colleagues, who directly linked gut microbiota dysbiosis and liver inflammation due to bacterial translocation with hepatic fibrosis in CCl_4-treated mice [17].

Furthermore, clinical and experimental data revealed different underlying pathogenic factors unveiling a particular importance of gut microbiota in hepatic disease. First, gut motility seems to be a decisive factor in disease development, as several studies have shown a reduced gastrointestinal motility in patients suffering from liver cirrhosis [18–20]. Various underlying causes have been proposed, including autonomic dysfunction, bowel wall edema, altered concentration of intestinal active peptides as well as changes in intestinal myoelectrical activity [21–23]. Small intestinal bacterial overgrowth (SIBO), which is causatively related to reduced gut motility and diminished gastric acid secretion, is another extensively studied condition that intensifies the influence of microbiota to liver disease [24]. SIBO is defined as a bacterial population in the small intestine exceeding 10^5 to 10^6 organisms/mL and shows a very high prevalence of 35–61 % in patients with cirrhosis [21, 24–27]. Chen et al. demonstrated a compositional change of intestinal bacteria in cirrhotic patients, showing a reduced proportion of Phylum *Bacteroidetes*, whereas the presence *Proteobacteria* and *Fusobacteria* were significantly increased [28]. Finally, changes in intestinal tight junctions integrity as well as impaired antimicrobial defence mechanisms, involving intestinal Paneth cells, contribute to an increased translocation of bacterial PAMPs and thus lead to the amplification of liver inflammation [29, 30].

9.3 Gut Microbiota and Hepatocellular Carcinoma

It has been well established that chronic hepatic inflammation followed by liver fibrosis and cirrhosis precedes hepatocarcinogenesis [31–33]. Having seen how gut microbiota affects the hepatic microenvironment in terms of a proinflammatory implication, a causal association between the development of hepatocellular carcinoma and the intestinal microbiota is rather obvious.

A growing number of animal models confirmed the assumed pathophysiological involvement of gut microbiota in the formation of hepatocellular carcinoma. Yu et al. analyzed the effects of microbial-driven endotoxins in a model of diethylnitrosamine (DEN)-induced hepatocarcinogenesis [34]. They observed decreased endotoxin levels as well as a subsequently significantly reduced tumor growth and multiplicity of HCC nodules in rats which received a bactericidal pretreatment with polymyxin B and neomycin or in mice which underwent genetic ablation of Toll-like receptor 4 (TLR 4). Conclusively, the group suggested that activation of TLR4 signaling by LPS in Kupffer cells of mice subjected to DEN treatment produces paracrine-acting, tumor-promoting cytokines that are not only capable of causing inflammation but also of stimulating the proliferation of adjacent premalignant hepatocytes [34]. Previous studies have identified IL-6 and TNF-α as major Kupffer cell-produced factors that amplify the growth of surviving DEN-initiated hepatocytes [34, 35].

These data were confirmed by the findings of Dapito et al. demonstrating that gut sterilization leads to a reduction of tumor number and size in mice subjected to a combination of DEN and the hepatotoxin carbon tetrachloride (CCl_4) [36]. Furthermore, the group demonstrated similar effects in mice that were kept in germ-free conditions and thereby excluded the notion that direct effects of antibiotics on the liver were responsible for HCC reduction. Conversely, long-term

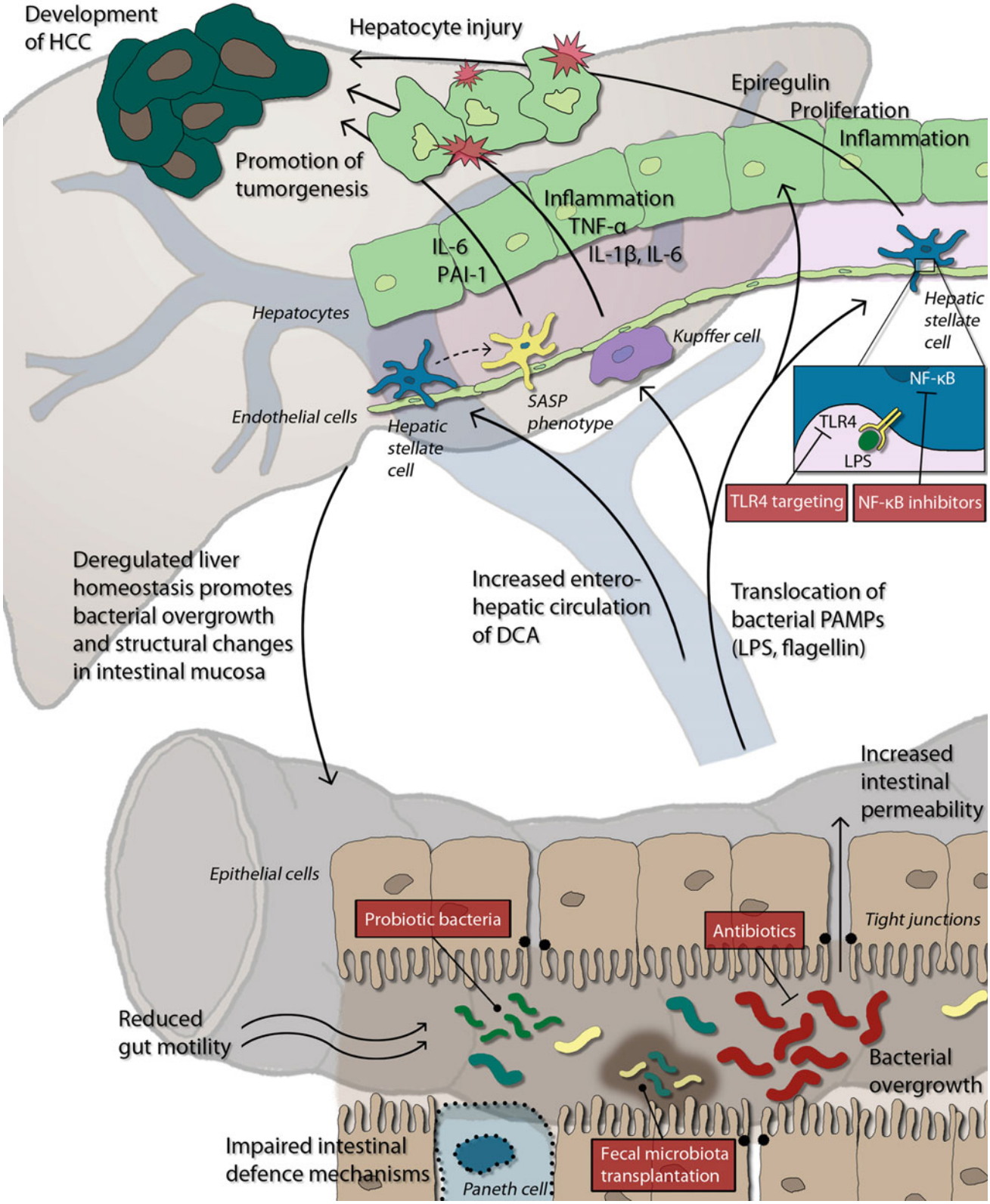
treatment with low-dose LPS led to a significant increase in HCC development. Looking at the molecular level, Dapito et al. [36] once more substantiated the determining role of TLR4 signaling. The study provides evidence that hepatocarcinogenesis in its early stage is particularly driven by TLR4-mediated secretion of growth factors such as epiregulin by hepatic stellate cells (HSC) [36]. Previous studies have shown the promotion of tumor development and growth factor signaling in an ECM-rich environment [37, 38], indicating that ECM and growth factors produced by HSC in the setting of hepatic inflammation are likely to synergize in tumor promotion [36]. NF-κB, a downstream molecule of TLR4 signaling, was found to be downregulated in all groups of mice being protected from HCC development by gut sterilization, genetic TLR4 inactivation, or germ-free status. Consequently, the number of cleaved-caspase 3-positive cells as a marker for apoptosis were increased in these mice [36]. Interestingly, the group only described a significant difference in tumor number and size between TLR4 mutant and TLR4 wild-type mice but did not manage to show a change in tumor incidences as it had been proclaimed previously by Yu et al. [34, 36].

Summarizing these findings, TLR4 signaling seems to be one of the major pathways in LPS-induced hepatocarcinogenesis. Toll-like receptors are a class of protein receptors that are involved in the recognition of PAMPs and play a major role in the regulation of inflammation [39, 40]. It is known that both Kupffer cells and HSC as well as hepatocytes express TLR4 and might thus function as the cellular link between microbiota-driven LPS and HCC development [41, 42]. Contrarily, Dapito and colleagues showed that only HSC and hepatocytes but not Kupffer cells are capable of mediating the tumor-promoting effects of LPS-induced TLR4 signaling, whereas Yu et al. demonstrated an essential role of hepatocytes and Kupffer cells in TLR4-dependent HCC development. Consequently, further studies are needed to finally elucidate the precise cellular and molecular mechanism of gut-driven LPS-induced hepatocarcinogenesis.

Sung and colleagues proclaimed recently that compositional changes in the gut microbiota may induce an enhanced induction of T_H17-cells through secretion of IL-23 by dendritic cells (DC) in the intestinal lamina propria [43]. Although the role of T_H17 cells in tumor immunity is still not fully understood, there are several lines of evidence suggesting a tumor-promoting role in HCC. An increase of T_H17-cells has been observed in tumor tissue [44, 45] and in peripheral blood [46] of patients suffering from HCC and T_H17 levels are correlated with poor disease prognosis [44–47]. Therefore, gut microbiota might be a potential source for tumor-associated T_H17 cells and could play a decisive role in modifying the local tumor environment by the secretion of IL-17, eventually leading to tumor progression [43].

Looking at the precise composition of gut microbiota, *Helicobacter hepaticus* has especially been associated with the induction and progression of hepatocellular carcinoma. In a murine model, Fox and colleagues were able to show that the intestinal colonization by *H. hepaticus* is sufficient to promote aflatoxin- and HCV transgene-induced HCC. *H. hepaticus* seems to incite transcriptional responses in the lower bowel and liver that converge on NF-κB-signaling and the activation of innate and Th1-type adaptive immunity without the requirement for bacterial colonization of the liver or the induction of hepatitis [48, 49]. On the other hand, a study published by Huang et al. demonstrated the presence of *Helicobacter spp.* in human liver samples from patients with primary hepatocellular carcinoma while being absent from healthy control samples [50]. Furthermore, Yang and colleagues reported a significantly higher rate of *H. hepaticus* infections in patients with primary HCC using serological and molecular biological detection [51].

As the prevalence of obesity in most developed countries has increased dramatically during the last decades and overweight in both adults and children has become one of the major public health burden of the twenty-first century, potential impacts on HCC development need to be considered. Different studies have shown that overweight and obesity are highly associated with the risk of cancer development [52–54]. Moreover, obesity seems to be accompanied by compositional alterations of intestinal microbiota and as a consequence might represent a major pathogenic factor for HCC development [55–57]. Yoshimoto et al. investigated the role of gut microbiota in an obesity related model of hepatocarcinogenesis. They demonstrated that reducing gut bacteria by the administration of antibiotics or blocking deoxycholic acid (DCA) production prevents the occurrence of HCC in obese mice after exposure to a chemical carcinogen [58]. DCA is a gut bacterial metabolite known to cause DNA damage and on this account has been associated with the pathogenesis of gastrointestinal cancer [59]. On a molecular level, the group showed that increased levels of DCA as a result of obesity-induced alterations of gut microbiota provoke a senescence-associated secretory phenotype (SASP) in hepatic stellate cells via the enterohepatic circulation of DCA, which in turn secretes various inflammatory and tumor-promoting factors in the liver such as IL-6 or PAI-1 [58]. Consistent with these results, the group showed that prolonged treatment of mice with DCA promotes HCC development in lean mice [60]. SASP is a cell-condition in which a senescent cell, originally determined to function as a barrier to tumorigenesis, turns into a proinflammatory cell with the ability to promote tumor progression [61, 62]. To summarize, an altered DCA-SASP axis in HSC due to adiposity-related changes in gut microbiota seems to play an essential role in obesity-induced HCC development.


Development of HCC
Hepatocyte injury
Epiregulin
Proliferation
Inflammation
Promotion of tumorgenesis
Inflammation
TNF-α
IL-1β, IL-6
IL-6
PAI-1
Hepatocytes
Kupffer cell
Hepatic stellate cell
NF-κB
TLR4
LPS
SASP phenotype
Endothelial cells
Hepatic stellate cell
TLR4 targeting
NF-κB inhibitors
Deregulated liver homeostasis promotes bacterial overgrowth and structural changes in intestinal mucosa
Increased entero-hepatic circulation of DCA
Translocation of bacterial PAMPs (LPS, flagellin)
Increased intestinal permeability
Epithelial cells
Probiotic bacteria
Antibiotics
Tight junctions
Reduced gut motility
Bacterial overgrowth
Impaired intestinal defence mechanisms
Paneth cell
Fecal microbiota transplantation

◀ **Fig. 9.1 The role of microbiota in the development of hepatocellular carcinoma**. Different structural and functional changes such as reduced gut motility, compositional changes in gut microbiota as well as small intestinal bacterial overgrowth (SIBO), impaired intestinal defence mechanisms and an increased intestinal permeability due to dysfunctional tight junction integrity lead to an enhanced bacterial translocation through the portal vein into the liver. LPS and other PAMPs produced by intestinal bacteria are capable of activating hepatic stellate cells and Kupffer cells, most likely by a TLR4 and NF-κB-dependent pathway. The activation of these cells results in the secretion of proliferative and proinflammatory chemokines causing severe hepatocyte injury and a tumorigenic hepatic microenvironment that enables HCC development. Furthermore, increased levels of deoxycholic acid as a result of obesity-induced alterations of gut microbiota provoke a senescence-associated secretory phenotype in hepatic stellate cells via the enterohepatic circulation of DCA, which in turn secretes various inflammatory and tumor-promoting factors in the liver. Possible future treatment options are displayed in the *red boxes* (IL-1ß/6: Interleukin-1ß/6, PAI-1: Plasminogen activator inhibitor-1, TNF-α: Tumor necrosis factor alpha, HCC: Hepatocellular carcinoma, SASP: senescence-associated secretory phenotype, DCA: Deoxycholic acid, LPS: Lipopolysaccharide, TLR4: Toll-like receptor 4, NF-κB: nuclear factor "kappa-light-chain-enhancer" of activated B-cells)

9.4 Therapeutic Options and Future Directions

Having seen the distinct involvement of gut microbiota in the pathogenesis of hepatocellular carcinoma by creating a LPS-dependent proinflammatory hepatic microenvironment, possible therapeutic treatment options in human disease need to be evaluated. As TLR4-signaling appears to be one of the key components of microbiota-associated hepatocarcinogenesis, a potential therapeutic approach in this signaling cascade is reasonable. Eritoran tetrasodium (E5564) binds to MD-2 [63, 64], a co-receptor of TLR4, thus inhibiting the activation of downstream signals and has been shown to limit inflammation induced by LPS and to improve survival in a model of sepsis [65, 66]. TAK-242 (resatorvid), a small-molecule inhibitor of the intracellular domain of TLR4, significantly decreased cytokine levels in mice undergoing LPS-injection and protected them from LPS-induced lethality [67]. So far, none of these potential therapeutic candidates have been tested in the setting of HCC prevention and therefore further evaluation in animal and clinical studies is needed. Furthermore Bortezomib, which prevents NF-κB nuclear localization and thereby could also diminish hepatocarcinogenic signaling, did not show any relevant activity in patients with unresectable hepatocellular carcinoma [68]. Attention should be paid to the fact that systemically antagonizing the innate immune signaling pathways might lead to an extended bacterial dysbiosis and could also result in enhanced proinflammatory gene expression. Thus, a general inhibitory modulation of TLR4 signaling should not be used without restrictions.

A more causative therapeutic approach is the targeted focused reduction of gut-driven proinflammatory stimuli to the liver. There are different therapeutic attempts to modify the composition of gut microbiota in order to reconstitute the altered intestinal microbial flora and restore the "leaky gut" state in chronic hepatic disease in order to diminish the amount of gut-driven proinflammatory stimuli to the liver. Probiotic microorganisms are known to restore the microbial equilibrium of the intestinal wall in patients with liver cirrhosis [69]. Zhang et al. observed a massively attenuated dysbacteriosis and intestinal inflammation as well as a decreased liver tumor growth and multiplicity in rats treated with the probiotic mixture VSL#3 [70]. Other probiotics such as various *Lactobacilli* strains [71, 72] or *Bifidobacterium CECT 7765* [73] were shown to reduce endotoxemia and inflammatory liver damage in different liver diseases such as NAFLD and alcoholic liver disease and thus might act as a future option to prevent HCC development in patients suffering from chronic hepatic disease. More specifically, probiotics seem to be capable of controlling the previously mentioned priming of T_H17 cells by DCs in the intestinal mucosa and thereby could diminish the tumorigenic microenvironment in the liver created by T_H17 after translocation to the liver [43].

Fecal microbiota transplantation (FMT) could also become a possible option for preventing or treating HCC. Shown to be an effective treatment option in severe and relapsing *Clostridium difficile* colitis, recent data demonstrated a broader applicability of FMT in metabolic diseases including NAFLD which is highly associated with hepatocarcinogenesis [74, 75].

Antibiotic gut sterilization seems to be an effective option to significantly reduce HCC tumor size and multiplicity in different rodent experimental models [34, 36]. Although the applied combination of antibiotics in these animal studies are not suitable for long-term treatment due to known side effects, rifaximin, a nonabsorbable antibiotic drug which is used to treat hepatic encephalopathy in patients with advanced liver disease [76], might be a potent candidate for HCC prevention as well. Dapito et al. showed a borderline significant reduction of tumor numbers without a significant reduction in tumor size in mice treated with rifaximin, suggesting only a moderate effectiveness of an antibiotic monotherapy. Thus, further studies are required to assess possible antibiotic combinations with reasonable side effects to reliably reduce HCC occurrence in patients with chronic hepatic disease (Figure 9.1).

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Hepatocellular Carcinoma as a Paradigm for a Systemic Evolutionary Approach to Cancer

10

Antonio Mazzocca, Giovanni Ferraro, Giovanni Misciagna, and Brian I. Carr

Contents

10.1 Introduction

The prevailing theory of cancer attributes its primary causes to mutations of nuclear DNA, such as oncogenes and tumor suppressor genes, [45]. Standard chemotherapeutic treatments in medical oncology are based in part on this assumption. However, this theory, which is often presented as dogma in textbooks of oncology, is in crisis [36]. Building even more elaborate genetic models of carcinogenesis has been linked to adding epicycle models to the pre-Copernican Ptolemaic paradigm of planetary motion in order to explain discrepancies in astronomical data without postulating that the earth revolves around the sun. The description of the motion of each newly discovered planetary body had to be retrofitted to Ptolemy's theory of "planetary perfection" [3]. A change of paradigm, from the genetic theory of cancer origin to a new theory, is now needed.

10.2 Prevailing Theories of Cancer

Many tentative theories of cancer have been suggested over the years and researchers have often developed unlikely and artificial divisions and grouping. Theories of cancer can be divided in many ways, e.g., in five groups or models (mutational, genome instability, non-genotoxic, Darwinian, tissue organization) [42] or in six groups (mutational, genome instability, Darwinian, epigenetic, tissue organization field theory, based on ontophylogenesis) [7]; or in three groups (tissue organization field theory, cancer stem cell theory, intrinsically disordered proteins) [39]. However, a simple division into two main groups [38, 40] can summarize all these different point of view:

A. Cellular theories of cancer.
B. Tissue theory of cancer.

The cellular theories include different subgroups that are updates of the initial somatic mutation theory of cancer, and

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are determined by new research findings: mutational standard theory, selection theory of cancer cell (Darwinian theory of cancer), mutator genes—chromosomal instability theory, epigenetic theory. The original mutational theory of cancer states that very few driver mutations in somatic cells are able to generate a cancer cell, and was initially based mainly on epidemiological and experimental studies [15], then supported by molecular biology studies with the discovery of oncogenes and cancer suppressor genes [45]. This theory has been modified to explain the heterogeneity of cancer cells, not only between different types of tumors or the same type of tumor between patients, but even within the same tumor in the same patient [9, 17]. To the somatic mutation theory of cancer pathogenesis (mutations generated in many different ways: X-rays, chemical substances, viruses, etc.) was added the concept of selection of the cancer cells that were most fit to compete with other cells to adapt to the environment [8]. Then, a new update of the somatic mutation theory was determined by the arrival of genomic data on cancer that showed that mutations in cancer cells are not few, but actually a huge number, so the theory was changed to include the concept of "mutator phenotype" resulting in a heterogeneous cell population), cells that have mutated genes that cause many contemporaneous or successive mutations, with chromosomal instability as a variant of this theory [4]. Finally, there has been another change of the somatic mutation theory, known as the epigenetic theory of cancer. This theory was proposed after the discovery that there are cancers without genetic mutations, which had only variation of intensity of gene expression or gene silencing, caused by the methylation or acetylation of histones or direct methylation of nuclear DNA [16].

A different theory is the tissue organization field theory, in which the cause of cancer is proposed to be a disturbed communication between different types of cells within their tissue of residence, caused above all by chronic inflammation [37, 41]. The theory of the pathogenesis of cancer cell as a consequence of a stem cell that does not evolve [35] can be considered in a certain way, as a subgroup of the field theory of cancer, or a compromise between field theory and somatic mutation theory. The updates to the somatic mutation theory and to the field theory, signal the fact that both theories probably are incomplete descriptions of cancer pathogenesis and a new theory is needed to explain cancer. There are certain cancer facts that are not explained by these theories of carcinogenesis, indicated as paradoxes in carcinogenesis [3], the most important of them being the cases of spontaneous regression of cancer. Furthermore, there are the findings from nuclear to cytoplasm transfer experiments that contrast with the somatic mutation theory of cancer origin [27]. We think that both the somatic cell mutation theory and the tissue organization field theory of carcinogenesis can be included in a new theory, a systemic evolutionary theory of the pathogenesis of the cancer cell that can better explain the conundrum of data on this disease.

10.3 Fundamentals for a New Theory of Cancer

There are some concepts from cellular evolution and systems biology that can be very useful to build a new theory of carcinogenesis.

10.3.1 Cellular Evolution

It is now clear that the formation of the eukaryotic cell is an exceptional event, due to the endosymbiosis of an archaea and a prokaryote more than 2 billions years ago [21, 24, 26]. These two very different types of bacteria started to collaborate, the archaea engulfing the prokaryote. The collaboration became so strict at a certain point that most of the genes of the prokaryote were transferred to the DNA of the archaea, saving a lot of energy of the primitive eukaryote [24]. The archaea (genetic material and cytoplasm) were able to metabolize glucose to pyruvate by anaerobiosis, generating a small amount of energy as ATP. However, the prokaryote (mitochondrion) was able to metabolize pyruvate to H_2O and CO_2, producing a major increase in quantity of energy per gene than the original pre-eukaryote, utilizing chemio-osmotic coupling and oxygen [23, 43]. What is really important about this endosymbiosis is not only the enormous increase of energy production per gene, that allowed increase of genome, synthesis of proteins (energetically more expensive than gene reproduction), and cellular evolution, but also the efficient elimination of metabolic waste. Instead of the lactic acid produced by the primitive archaea, the eukaryote produced the easily eliminable H_2O and CO_2, a very efficient way to eliminate the waste generated by an increased consumption of energy, a wonderful system design of the eukaryote cell that could also allow for multicellularity [5].

10.3.2 Systems Biology

The eukaryote can be conceptualized [28] as an emergent system made by two subsystems. One subsystem produces information and little energy (the old archaea, now the nucleus and cytoplasm), while the other one produces energy and little information (the old prokaryote, now mitochondrion) with the waste of the first subsystem (lactate) managed by the second subsystem to become CO_2 and H_2O, in an almost perfect system design [5]. This way to look at the cell from the systemic point of view, using the

concepts of boundaries, hierarchy of systems and emergence, is quite different from the concept of a cell as a network (a reductionist way to think of systems) shown in many textbooks of systems biology of the cell. This systems thinking is more like the systems approach in the area of the theory of carcinogenesis [31], but applying systems thinking to tissues, while our new theory starts from the cell itself before considering tissues. The two subsystems of the eukaryote, the informative and the energetic one work in series, even though the energetic subsystem is made by many copies of the same unit (the mitochondrion) that work in parallel to safeguard the energy production for the cell. However, the plurality of mitochondria can be considered as a single subsystem with its own boundary from our modeling point of view. The eukaryotic cell as a complex adaptive dynamical system emerges from the symbiogenesis (endosymbiosis) of these two subsystems, the archaea and the prokaryote, in a new boundary (the cell membrane). This endosymbiosis generates a nonlinear change of the merged activities: information concentrates, energy multiplies, and wastes are more manageable from the environmental point of view. All these characteristics open the way to the evolution of the primitive eukaryote to a complex adaptive dynamical system, completely different from the previous single archaea and prokaryote, even though including them.

10.4 A Systemic Evolutionary Theory of Cancer

The systemic evolutionary theory of cancer pathogenesis states that cancer is generated by the de-emergence of the eukaryotic cell system and by the reappearance of its archaea and prokaryotic subsystems, with autonomous, or at least uncoordinated, behaviors, a hypothesis suggested by very few authors [2, 10]. This de-emergence of the eukaryote generates problems at cell and tissue level, and eventually it can threaten the survival of the whole organism. A first step in cancer pathogenesis is a decrease in coordination between the two subsystems of the eukaryotic cell, the archaea (nuclear DNA and cytoplasm) and the prokaryote (mitochondria) that begin to work independently. This decreased coordination can be caused by a change in the organization of the eukaryote environment, mainly inflammation [6]; a damage to mitochondrial DNA and/or to its membrane composition [33] determined by viruses, chemicals, hydrogenated fatty acids in foods, etc.; a damage to nuclear DNA that control mitochondria energy production or metabolic pathways like glycolysis [44]. In all these cases, the final result is the de-emergence of the eukaryote, with the reappearance of its old subsystems, the archaea and the prokaryote, which now work separately. This systemic change allow the de-emerged cell to survive, but at the expense of the surrounding cells and the organization of the tissue, and at the end, of the whole organism. There are quantitative and qualitative changes in the de-emerged eukaryote, mainly in its way of producing energy, eliminating waste, and interacting with other cells [1], that make the cell assume "atavistic" characteristics. These phenotypic changes can be determined by the somatic mutation of single genes in series, one after the other, or by the simultaneous change of many genes caused by a driver-mutator gene. However, this change of functions (reappearance of old gene organizations present in the ontophylogenesis of the organism) is better determined by the simultaneous and coordinated change of many gene networks under the pressure of the de-emerged eukaryotic cell struggling to survive in a new cell organization and/or environment. The hallmarks of cancer [18], the Warburg effect [34], cancer glutaminolysis [11], the adaptations of the cells surrounding the cancer cell metabolizing lactic acid, a sort of eso-symbiosis to substitute the failed endosymbiosis [32] are all characteristics of the cancer cell. This could be reinterpreted in the light of the de-emergence of the eukaryotic cell (in the light of evolution) and its association with changes in many nuclear gene networks. They are consequences of the uncoordinated functioning within the cell membrane boundary of the nucleus-cytoplasm (archaea) subsystem and of the mitochondria subsystem (prokaryote).

The second step of cancer pathogenesis, including dissemination of cancer cells (metastasis) may be supported by a decrease in mitochondrial functionality below a certain threshold, in association with a simultaneous increase in the activity of the anaerobic part of the eukaryotic cell: the nucleus-cytosol [25, 46]. Nuclear and mitochondrial genetic mutations and tissue inflammation can determine the neoplastic transformation of the eukaryotic cell, but the real explanation for the pathogenesis of cancer is a systemic change at the cellular level: the de-emergence of the eukaryote cell, and its division into the old archaea and prokaryote within the same boundary, with consequent changes in the management of energy and waste, and relationship with other cells. These cellular changes cause modifications at the tissue level [19], and then at the organism level. It is this de-emergence of the eukaryotic cell which is the primary cause of cancer and that makes many gene networks change at the same time. Only a systemic evolutionary theory of cancer can explain the transformation of a normal cell, a complex adaptive dynamical system, in a cancer cell, another complex adaptive dynamical system, but selfish and uncoordinated with the other cells in the tissue of origin. The proliferation of cancer cells can be stimulated (promoted) by the modern diets, rich in carbohydrates and animal proteins that feed anaerobic glycolysis with sugar and mitochondrion with proteins [13].

10.5 Hepatocellular Carcinoma as an Application Model to the Systemic Theory of Cancer

Hepatocellular carcinoma (HCC) is estimated to become the third leading cause of cancer-related deaths by 2030 in the United States [30]. But incidence and epidemiology apart (illustrated exhaustively elsewhere: El-Serag and Rudolph [14]), HCC is an excellent model for studying the pathobiology of cancer. This is because HCC normally develops in a liver with chronic disease, generally hepatitis and/or cirrhosis. Therefore, HCC is an example of a multistep pathogenesis of cancer where determinant risk factors such as inflammation, regeneration, and fibrosis represent the background for HCC development. The fact that hepatocarcinogenesis is strongly related to chronic liver disease has also been largely shown by epidemiological studies [20]. HCC development requires several steps leading to the acquisition of tissue, cellular, and molecular alterations necessary for cell transformation. The natural history of disease usually involves a chronic hepatitis (often viral), which represents an important risk factor. The evolution of this condition to a fibrotic or cirrhotic liver, with alteration of the hepatic tissue architecture and vasculature, predispose to dysplastic or pre-neoplastic areas and nodules. These are the hotbeds where HCC develops. This is accompanied or associated with genetic (generally, the frequency of replication errors is low in HCC whereas there is a high prevalence of chromosome abnormalities) or epigenetic (that seems to have a predominant role during the long pre-neoplastic stage and the early phases of HCC development) modifications. However, little attention has been paid on the plasticity of hepatocytes (as an integrated cellular system) during the long and stepwise process of carcinogenesis considering, for example, the availability of energy and/or oxygen in the hepatocyte during cancer transformation. In other words, when do the two subsystems, the archaea and the prokaryote (see above) work as a coupled system? This question offers an important starting point on why hepatocarcinogenesis is a valuable model to support the systemic evolutionary theory of cancer. The availability of energy is a suggestive explanatory link between the multistep development of HCC and the aforementioned theory, because the chronic damage to the liver may offer an interesting model to the depletion of energy [12]. Here, we propose that an energy package is constantly required by the hepatocyte to maintain its differentiated status. Normally, in the absence of tissue alterations, this is constantly guaranteed. In particular, we postulate that in normal conditions, when the energy flow works properly, the two subsystems (the archaea and the prokaryote) are perfectly integrated and there is no prevalence of one system on the other. As a consequence, the hepatocyte maintains its differentiated status. As soon as injury is applied and the liver becomes damaged, the flow of energy is restricted, but is still in a condition to recover if the liver damage does not last long. However, if the damage lasts long (i.e., chronic inflammation), then the energy package (amount of energy) necessary to maintain the cell differentiation can be reduced in level and this may overtime cause the gradual decoupling of the two subsystems. When the energy package becomes constantly insufficient the two subsystems get completely uncoupled, with the "prokaryote" subsystem becoming predominant. In the cirrhotic liver, this process can be favored by the alteration of the oxygen availability due to the altered vasculature and the fibrotic barrier. The prevalence of the "prokaryote" subsystem may explain the metabolic alterations (i.e., glucose, other metabolic pathways) seen in neoplastic cells as well as the capacity for proliferation and invasion, especially toward areas of major oxygen availability (i.e., alteration of blood vessel architecture, arterialization of portal vein, etc.). The scenario proposed here may also explain why tissue integrity is essential to constantly guarantee the availability of a given amount of energy required for maintaining the status of differentiated cell. Thus, tissue integrity is essential for the proper flow and availability of energy, and therefore, for the maintenance of cellular homeostatic functions. When integrity is not maintained over time, the balance is broken and the two subsystems become imbalanced in favor of the prokaryote. In the process of neoplastic transformation this becomes particularly evident and could be one of the mechanisms that supports hepatocarcinogenesis.

10.6 Conclusions

The systemic evolutionary theory of cancer pathogenesis can open the way to new cancer treatments. One of these new treatments could be a ketogenic diet [29], controlling mainly the archaeal subsystem of the cancer cell, the uncontrolled self-reproducing nucleus and the cytoplasmic aerobic glycolysis. Intracellular antibiotics could be added to this type of diet (i.e., macrolides) to control the prokaryotic component, the still working mitochondria [22]. This new therapeutic approach to cancer treatment could be also a test of the systemic evolutionary theory of cancer pathogenesis, the de-emergence of the eukaryote as a primary cause of cancer.

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HCC and Its Microenvironment

11

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Contents

11.1 Causes of HCC

HCC typically arises on the basis of a chronically inflamed liver, most often due to cirrhosis. The commonest causes are HBV, HCV, alcoholism, nutritional mycotoxin exposure (Aflatoxin B1), obesity or a combination of them. Thus, most HCC patients have two diseases of the liver simultaneously: cirrhosis (from many causes), which is potentially hepatocarcinogenic (pre-malignant), as well as HCC.

11.2 Gene Expression Profiles in the Tumor and Surrounding Tissues

The idea that the chronically inflamed, cirrhotic liver is not only a precursor and predictor of risk for HCC, but also may influence the biology of HCC, is relatively new. Several studies have shown that the non-HCC liver has prognostic significance and may also predict recurrence after primary treatment and begun to identify HCC phenotype subclasses [1–11]. These studies show that although genetic studies of the tumors identified various HCC subtypes and their associated tumor biologies, the non-tumor underlying liver does so even more. This has focused attention on the composition of the tumor and specifically the HCC microenvironment, and also on the role of the microenvironment on influencing HCC behavior and aggressiveness (tumor mass, multifocality, portal vein invasion, metastasis), and the mechanisms underlying these processes. Furthermore, this tumor/microenvironmental cross-talk [12] has been found to be reciprocal with each influencing the other (reviews: [13–16]).

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11.3 Composition and Actions of the Microenvironment in Presence of HCC

The above observations of the interaction of HCCs and their surrounding tissue (microenvironment), coupled with the growing awareness that the neovascularity was a requirement for supporting increased tumor growth in size beyond a minimal point, focused attention to the composition of the microenvironment and its functions, with respect to HCC growth, invasion, and metastasis. The microenvironment can be broadly viewed as having a cellular and a noncellular composition (Fig. 11.1).

The cellular components include stromal cells, endothelial cells, tumor-associated macrophages (TAMs), immune cells, neutrophils, and platelets, amongst others. Stromal cells (carcinoma-associated fibroblasts or CAFs, hepatic stellate cells or HSCs) produce stromal collagen through the effects of stromal cell derived factor 1 and CXCL12, which in turn contribute to HCC growth tumor angiogenesis. It is also becoming clear that microenvironmental factors are involved, both in the normal liver immunity, and in the suppression of that immunity that permits HCC growth. The inflammatory microenvironment, both consequent on chronic hepatitis or other causes of cirrhosis, or in response to the growth of the HCC, has recently been shown to be important in influencing HCC biology and patient prognosis [15–18]. So has systemic inflammation [19]. Furthermore, attempts to decrease inflammation appear to be associated with decreased HCC in those at risk [20]. In fact inflammation scores correlate with prognosis in many tumors, including HCC [21].

11.4 Microenvironmental Platelets and HCC Growth

Cirrhosis is often associated with portal hypertension, dependent on the degree of hepatic fibrosis, with consequent splenomegaly and thrombocytopenia. This thrombocytopenia has even been used as a surrogate for providing evidence of cirrhosis [22]. It has been shown that thrombocytopenia-associated HCCs tend to be small in size [23, 24]. By contrast, thrombocytosis is associated with more aggressive tumors in general [25, 26], and with larger sized HCC [27, 28]. Experimental evidence supports the idea that platelets may play a direct role in the growth of larger size HCCs [29], likely due to their α granules containing the growth factors EGF, PDGF, IGF-1, serotonin, and FGF, all of which have been previously shown to be HCC mitogens, in addition to their content of inflammatory cytokines. Furthermore, inhibitors of platelet action, aspirin and clopidogrel (inactivator of the ADP-receptor P2Y12) have been shown to antagonize experimental hepatocarcinogenesis in vivo [30, 31].

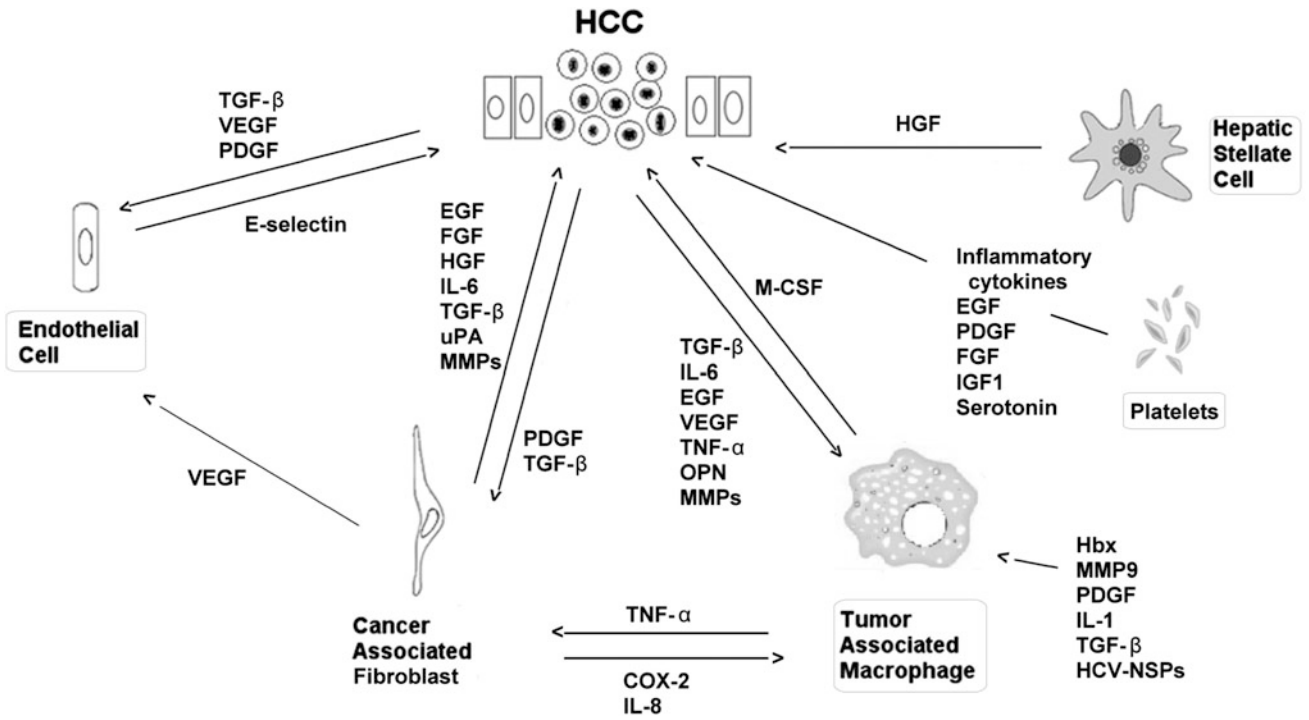

Fig. 11.1 HCC and its microenvironment

11.5 Microenvironmental Platelets and Modulation of Effects of Anti-tumor Drugs

It has recently been shown that human platelet lysates antagonized the inhibitory effect mediated by Sorafenib or Regorafenib on cell growth and motility in several HCC cell lines [32]. The molecular pathways involved in this interference were explored and it were found to include an increase by platelet actions in p-ERK levels, as well as the phosphorylation levels of its downstream targets p38 and STAT3, thus antagonizing the growth inhibitory effects mediated by Sorafenib or Regorafenib. These kinases are considered to be important molecules in mediating cell proliferation, but they are also involved in the modulation of anti-apoptosis mediators such as Bcl-XL and surviving. Platelets were also found to counteract the drug-mediated induction of apoptosis by decreasing the levels of pro-apoptotic BIM and Bax. Experiments designed to elucidate the role of platelet lysates in the modulation of migration and invasion, showed that they antagonized multikinase inhibitor-mediated inhibition of cell motility [30]. Furthermore, platelets were found to modulate the cytotoxic chemotherapy effects of doxorubicin on HCC cells [33]. Increased levels of P-JNK and P-p38 were found in HCC cells exposed to platelet lysates, in comparison to the levels of the same molecules in doxorubicin-treated cells. Both of these signaling kinases could be considered mediators of the protective role exerted by platelets; however, they can also be considered to be pro-survival mediators, depending on cell line and drug [34]. Others have shown in other tumor types that platelet alterations and microenvironmental alterations can modulate sensitivity or resistance to anti-cancer therapy [35–38]. There are many mechanisms that might mediate this modulation of drug sensitivity [39]. One mechanism derives from the many growth factors in platelets. Recent findings in HCC indicate that both EGF and IGF1 [39–41] have the ability to increase dug resistance. The opposite should still be also true, namely that growth factor inhibitors and platelet inhibitors may well increase the sensitivity of cancer cells to anti-cancer therapies (review: [42]).

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Circulating Tumor Cells (Liquid Tumor Biopsy) in Hepatocellular Carcinoma: Biology, Methodologies, and Clinical Implications

12

Zhengfeng Yin

Abbreviations

aCGH	Array comparative genomic hybridization
AFP	Alpha-fetoprotein
Akt	Protein kinase B
ASGPR	Asialoglycoprotein receptor
BCLC	Barcelona clinic liver cancer
bFGF	Basic fibroblast growth factor
CAM	Collagen adhesion matrix
CCSCs	Circulating cancer stem cells
CEA	Carcinoembryonic antigen
CGH	Comparative genomic hybridization
CICs	Cancer-initiating cells
CKs	Cytokeratins
CPS1	Carbamoyl phosphate synthetase 1
CSCs	Cancer stem cells
CT	Computer tomography
CTCs	Circulating tumor cells
CTM	Circulating tumor microemboli
DAPI	Dye 4′,6-diamidino-2-phenylindole
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
ELISPOT	Enzyme-linked immunospot assay
EMT	Epithelial–mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
EPISPOT	Epithelial immunospot
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration
FISH	Fluorescence in situ hybridization
GFP	Green fluorescent protein
HBV	Hepatitis B virus

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HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HER2	Human epidermal growth factor receptor-2
IGFBP1	Insulin-like growth factor-binding protein 1
IL-6	Interleukin-6
IL-8	Interleukin-8
ISET	Isolation by size of epithelial tumor cells
MACS	Magnetic activated cell sorting
MET	Mesenchymal–epithelial transition
MICs	Metastasis-initiating cells
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
PBMCs	Peripheral blood mononuclear cells
PET	Positron emission tomography
PI3K	Phosphoinositide 3-kinase
PSA	Prostate-specific antigen
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
PVTT	Portal vein tumor thrombus
RBCs	Red blood cells
RFS	Recurrence-free survival
SNP	Single nucleotide polymorphisms
TACE	Transcatheter arterial chemoembolization
TERT	Telomerase reverse transcriptase
TNM	Tumor-node-metastasis
WBCs	White blood cells

Contents

12.1 General

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer in men and the seventh in women worldwide, and the incidence is rising, with roughly 700,000 cases diagnosed globally in 2012 alone [104, 177]. HCC usually occurs in the setting of liver cirrhosis developed during a long process of inflammation and fibrosis, because of chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections, heavy alcohol consumption, nonalcoholic steatohepatitis, exposure to aflatoxin B1, obesity associated with fatty liver disease, primary biliary cirrhosis, or alpha1-antitrypsin deficiency [29, 50, 146]. HCC is also one of the most aggressive cancers, and currently listed as the third leading cause of cancer-related death. If the tumor cannot be completely removed, the disease is ultimately fatal within 3–6 months [29, 146]. In carefully selected patients diagnosed at an early stage, surgical resection, liver transplantation, and local ablation are potentially curative for HCC, with 5-year survival rates of 60–80 % for resection, 53–73 % for transplantation, and 40–70 % for ablation [29, 50, 112, 113]. However, surgical resection, liver transplantation, and ablation are associated with postoperative recurrence rates of 50 % at 3 years, 18 % at 3 years, and 70 % at 5 years, respectively [27–30, 50, 106, 112, 113, 123], that jeopardize overall survival in these patients, and finally lead to death in almost all patients. In liver transplantation for HCC, the most common site of an early posttransplant recurrence is the transplanted allograft [112, 113, 123]. This fact strongly suggests that cancer cells in the peripheral blood circulation are really an active source of HCC recurrence or metastasis. Tumor cells that are shed into the bloodstream from solid tumor origin are referred to as circulating tumor cells (CTCs) [211]. Animal experiments with human HCC xenografts have shown that CTCs are continuously released from the primary tumor into the bloodstream [52, 155, 156]. CTCs may spread to and deposit in multiple distant organs and initiate metastases. In the case of liver resection or transplantation, they may return to the liver remnant or the newly implanted healthy liver and initiate intrahepatic recurrence.

Although CTCs were discovered more than a century ago [12], research on CTCs has only recently become a very active field largely because they can now be efficiently isolated. CTCs bear an especially great potential to improve our understanding of steps involved in the metastatic cascade, starting from intravasation of tumor cells into the circulation until the formation of clinically detectable metastasis. As a novel biomarker for the metastatic disease process, CTCs essentially provide a readily accessible and real-time liquid biopsy of tumors to replace biopsies of metastatic tissue. The enumeration and characterization of CTCs hold great promise for the diagnosis of patients with early cancer or early recurrence, the identification of patients at a high risk for local or systemic relapse, the stratification of patients to specific therapies, and the monitoring of response or resistance to therapeutics [13, 76, 131]. This chapter describes recent discoveries related to the biology of CTCs that illustrate how they are involved in hematogenous spread, discusses the role of CTCs as a minimally invasive liquid biopsy for real-time identification of specific markers, outlines their potential clinical utility, reviews the current state-of-the-art on different techniques or strategies available for the detection and isolation of CTCs (with a special focus on HCC CTCs), primarily introduces recent progress obtained from clinical studies on detection of CTCs and circulating cancer stem cells (CCSCs) in HCC patients, proposes some strategies targeting CTCs for the management of HCC aiming to prevent postoperative recurrence and metastasis, and finally presents upcoming challenges and future perspectives on CTCs as a biomarker in precision therapy for HCC.

12.2 The Biology of CTCs

Solid tumors vary widely in their ability to shed CTCs into circulation. CTC levels are different across tumor types, even with a given tumor type [13, 76, 131, 211]. It appears that only a very small fraction of CTCs gives rise to distant metastases, a phenomenon regarded as “metastatic inefficiency” [39, 193]. However, the biology of CTCs remains poorly understood. For example, how CTCs disseminate, survive in circulation, and avoid apoptosis and host immunity, and home to different distant organs as sites for potential metastasis have yet to be elucidated.

12.2.1 Epithelial–Mesenchymal Plasticity

The metastatic process is comprised of the following steps: local invasion; intravasation; hematogenous survival and transport; extravasation; and colonization [38, 80, 173] (see Fig. 12.1, [98]). Cancer cells are of epithelial origin. Epithelial–mesenchymal transition (EMT) in cancer cells is a highly complex dedifferentiation program, and thought to be responsible for various steps in the metastatic cascade. Acquisition of the EMT phenotype by cancer cells enhances their migratory and invasive properties, and thereby enables them to enter the circulation by traversing the basement membrane, interstitial spaces, and blood vessels. During EMT, typical epithelial markers such as epithelial cell adhesion molecule (EpCAM) and E-cadherin are downregulated, keratin expression is altered, and finally, mesenchymal markers such as vimentin are upregulated [142, 173, 203]. The characterization of CTCs demonstrated the presence of a number of cells with EMT phenotype in certain tumor types.

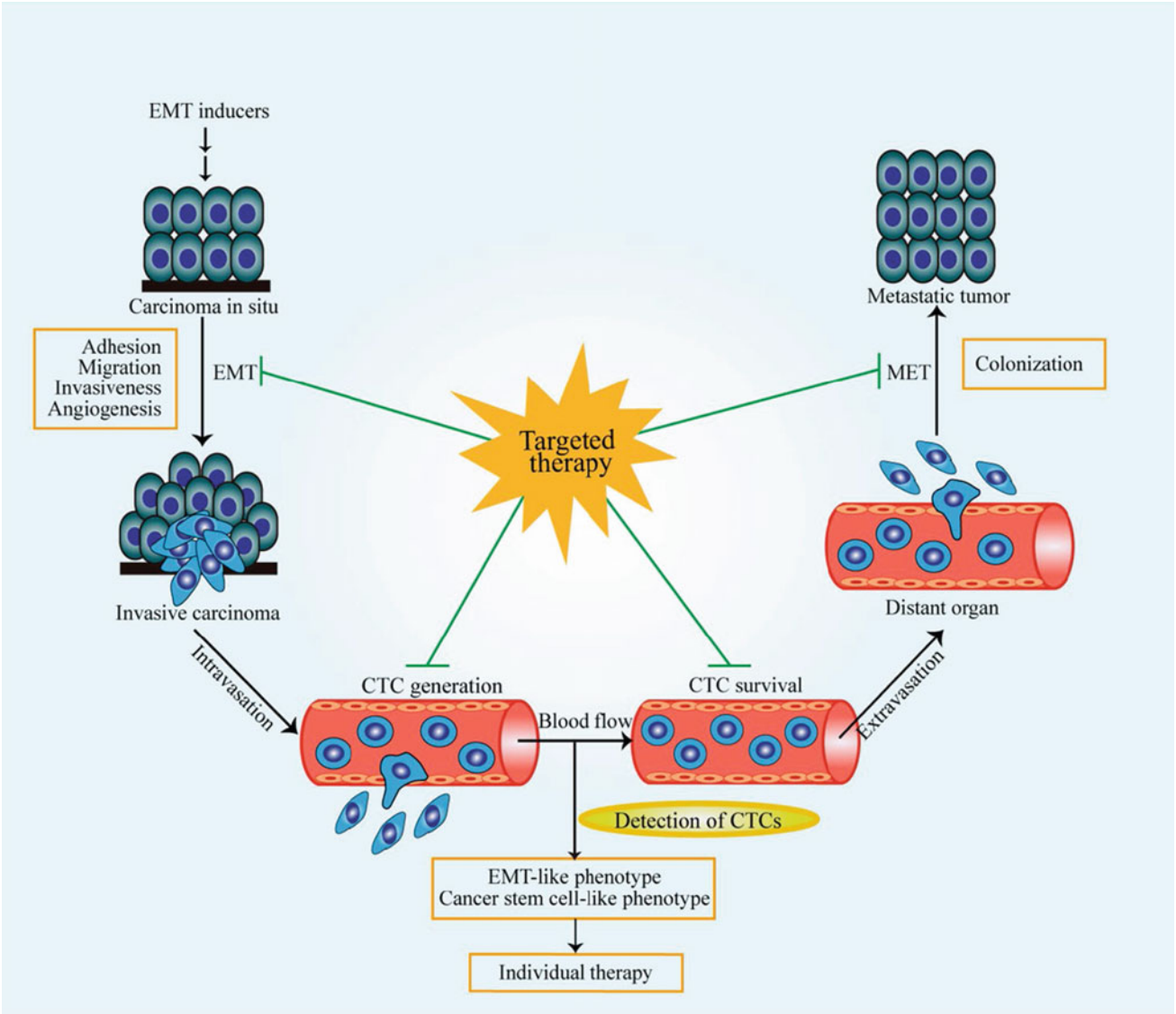

Fig. 12.1 The role of the epithelial–mesenchymal transition (EMT) and the mesenchymal–epithelial transition (MET)-targeted therapy during the individual steps of tumor metastasis. Metastasis is presented as a contiguous and complex process that depends on (1) local invasion, (2) intravasation, (3) hematogenous survival and transport, (4) extravasation, and (5) colonization. Circulating tumor cells (CTCs) represent an essential bridge for the metastatic cascade. EMT is involved not only in the generation of CTCs, by enhancing migratory and invasive properties of cancer cells, but also in their survival in the bloodstream. Subsequent activation of MET is responsible for extravasation of CTCs and colonization in distant organs, which ultimately forms the metastatic lesion. Therefore, the potential to effectively target EMT/MET processes during the individual steps of tumor metastasis may represent a promising approach to alleviate cancer metastasis and inhibit recurrence. In addition, detection of EMT phenotype-based subsets of CTCs in cancer patients may serve as a novel diagnostic tool for prognosis and individualized treatment. Reproduced from Liu et al. [98]. Permission from Springer

Once inside the bloodstream, CTCs face several natural obstacles caused by exposure to the poor survival environment that hinder the metastatic process. For instance, blood flow generates the enormous mechanic shearing forces and collisions with blood cells, which dramatically decrease the number of viable cancer cells. Compared to epithelial tumor cells, CTCs that underwent EMT seem to be more resistant against these forces [120]. Experimental data also suggest a continuum development of CTCs, ranging from pure epithelial to pure mesenchymal phenotypes, or to a partial EMT state, thus being able to switch between epithelial-like and mesenchymal-like cells [142, 173]. The population of cells with this "phenotypic plasticity" is characterized by stem cell-like properties and increased resistance to chemotherapy and targeted therapy, thus being determinant of aggressive behavior and more dangerous than cells in

pure epithelial or pure mesenchymal states [142, 173]. The mesenchymal phenotype of CTCs that underwent EMT promotes motility, but does not favor growth [37, 72]. Recent experimental studies have provided evidence for the functional requirement of EMT reversal in CTCs for the final step in the metastatic cascade. Cancer cells must undergo a reverse process, known as mesenchymal-epithelial transition (MET), to reacquire the ability to proliferate and thus colonize after they have settled down in the secondary organs [19, 171].

In summary, as shown in Fig. 12.1 [98], acquisition of the EMT phenotype by cancer cells not only facilitates their abilities of migration and invasion, thereby promotes their infiltration of the vascular endothelium and migration into the circulation, and finally generates CTCs, but also enhances their survival in the bloodstream and extravasation out of the circulatory system and invasion into proximal tissues. In distant organs, MET activation enables CTCs to grow and recolonize in the new microenvironment, ultimately forming a metastasis [173]. Therefore, the subset of CTCs switching between epithelial and mesenchymal phenotypes is thought to be an optimal target for precision medicine research, offering new hope for alleviating cancer metastasis and recurrence.

12.2.2 Circulating Cancer Stem Cells (CCSCs)

Increasing studies provided evidence that human cancers contain a small population of cancer stem cells (CSCs) with cancer initiation capacity. So CSCs, also known as cancer-initiating cells (CICs), may thus represent the major target of new drugs in clinical trials [142]. Given that CTCs arise from the tumor and contribute to metastasis, one could speculate that CTCs may comprise a minor subset of CCSCs. “Metastatic inefficiency” is an ancient concept, and has been in part demonstrated through experimental studies that only approximately 0.01 % of cancer cells injected into the circulation form metastatic foci [193]. Accordingly, several clinical studies have revealed that aggressive cancers release thousands of cancer cells into the bloodstream each day [5, 14, 124, 193], but most patients develop only few metastases, also suggesting a highly inefficient process of metastasis. For aggressive cancer cells, leaving a tumor appears to be relatively easy. “Not all detected cells are bad and not all bad cells are detected” [195] implies that although directly involved in the metastatic cascade, not all subpopulations of CTCs are likely to have the same metastatic potential, and only few CTCs are characterized by metastatic potential and high biological aggressiveness. This very small fraction of CTCs is supposed to be composed of CCSCs, and also renamed “metastasis-initiating cells” (MICs) [18]. In fact, accumulating evidence shows that a majority of CTCs with a stem phenotype circulate in blood of patients with various types of cancer and are potentially the most dangerous, with the capabilities of self-renewal, multipotency, and relapse or metastasis initiating. For example, CTCs in HCC that displayed an ICAM-1(+) or EpCAM(+) or CD90(+) surface phenotype possess CSC features such as tumor induction and sphere-forming capacities [101, 168, 205, 206]. Therefore, the characterization and eradication of these CCSCs should become one of the main goals in CTC research.

CSCs might be derived from either differentiated progenitor cells or somatic stem cells. Two hypotheses have been proposed to understand the origin of CCSCs. One is that CCSCs may arise from fully differentiated cancer cells that acquire migratory and invasive properties due to the activation of EMT pathways [23]. Another possibility is that cancerous somatic stem cells underwent EMT, also called mesenchymal CSCs, and migrate from the primary tumor into the blood circulation. When undergoing MET, they become epithelial stem cancer cells [145]. Whatever is the way, CCSCs are directed toward a niche through intermediary cells with an increased epithelial–mesenchymal plasticity and mobility [19, 72, 171]. Actually, data from multiple studies show that a small subpopulation of CTCs bears the variety of epithelial, mesenchymal, and stem cell-like markers, and are endowed with stemness characteristics following the EMT [2, 8, 90, 134, 137, 208]. Therefore, it is likely that the so-called MICs arise from CCSCs with an EMT phenotype [23, 145, 195], and will be a valuable therapeutic target for the eradication of cancer.

12.2.3 Anoikis

When dissipating from their tissue origin, cells from solid tissue are normally programmed to undergo anoikis, a kind of programmed cell death induced by loss of substrate adhesion or by inappropriate cell adhesion. Anoikis has functions to maintain the balance between proliferative potential of normal cells to ensure tissue integrity [133]. For aggressive cancer cells, leaving a tumor appears to be relatively easy, and a large number of cancer cells are released into the bloodstream each day [5, 14, 124, 193]. After leaving the primary location and intravasating into the circulatory system, cancer cells should survive in a complete absence of extracellular matrix (ECM). However, CTCs are fragile, and most in the circulation may die. The half-life of CTCs in the bloodstream is only 1–2.4 h [116]. One of the main causes of their fragility is their susceptibility to anoikis [31]. It is not surprising that a high proportion of CTCs originating from various types of cancer show signs of apoptotic cell death [6, 79, 150, 151, 163, 165]. Thus, anoikis is a potential barrier to their metastasis. In order to

survive during the circulation and spread, cancer cells must establish mechanisms or signaling pathways for anoikis resistance [21, 68].

12.2.4 Circulating Tumor Microemboli (CTM)

CTCs can be present in the circulatory system in various forms, such as simple cells and cell clumps. The latter may be composed of at least 2 cancer cells up to large microemboli with more than 50 CTCs and have also been referred to as circulating tumor microemboli (CTM) or clusters of CTCs [56, 67, 86]. The generation of CTM is supposed to be resulted from intravasation of cancer cell clusters via a leaky vessel in the primary tumor or from their aggregation in circulation due to their collective migration and adhesion [1, 41, 66]. In order to maintain the cell-cell contact, CTM may contain accessory host cells besides cancer cells, such as platelets, leukocytes, cancer-associated fibroblasts, endothelial cells, and pericytes [86, 88]. This configuration of clusters not only leaves the innermost cells protected from anoikis, immune surveillance, and the stresses of circulation, but also presents a favorable microenvironment for cancer cells to crosstalk and survive [66, 67]. The significance of CTM has been debated for a long time, and even the question has been discussed that such clusters may be merely artifacts of sample processing. However, the role of CTM in metastasis development was recently emphasized. An animal study showed that CTM were indeed cancer cell clumps breaking off from the primary tumor, and derived from oligoclonal groupings of primary cancer cells held together through plakoglobin-dependent intercellular adhesion, not from intravascular aggregation events [1]. When intravenously injected in vivo, cell clusters had a higher tendency to seed distant metastases than single cell, and larger sized CTM could form more metastatic foci than an equal number of smaller CTM [1, 88, 174, 176]. In clinical studies, several groups have observed CTM in the circulation of patients with several types of advanced cancer and absence of apoptotic cells within CTM. Moreover, the presence of CTM was significantly associated with a worse prognosis [25, 34, 56, 66, 82, 110, 121, 167, 185]. In liver cancer, patients with CTM displayed significantly shorter survival than patients without CTM [185]. The possible explanations for the increased metastatic potential of CTM include: (1) Larger CTM are more easily trapped in narrow blood vessels than individually CTCs, thus favoring extravasation into distant organs; and (2) CTM provide a favorable "their own soil" for cancer cell survival.

Collectively, the current knowledge for CTM is incomplete due to their extremely low abundance. Further studies require sensitive and specific methods for detection of CTM, thereby we can identify the cell composition and microenvironmental effect, and explore whether CTM are a better biomarker for increased metastatic potential than single CTCs.

12.2.5 Tumor Self-Seeding by CTCs

CTCs not only can seed metastases in distant organs, but can also preferentially return to and grow in the primary tumor. This phenomenon was described in a landmark study by Kim et al. [84] and termed "tumor self-seeding by CTCs." The mechanisms underlying this process are less well understood. In the study of Kim et al. [84], inflammation was identified as a major driver of tumor self-seeding by CTCs, as evidenced by that the tumor-derived interleukin-6 (IL-6) and interleukin-8 (IL-8) acted as attractants for CTCs, and "infiltrative" genes expressed by the attracted CTCs, such as collagenase-1/metalloproteinase-1 as well as the actin cytoskeleton component fascin-1, acted as mediators of their infiltration into primary tumors.

After primary tumor surgery, patients may have a constant population of CTCs, and tumor self-seeding by CTCs likely also occurs at remote metastatic sites. Taking liver cancer for an example, even though a localized lesion is completely resected, a new cancerous lesion may recur in the original site after a period of time. Besides, after liver transplantation, tumor reseeding may still occur in the explants (implanted grafts). For instance, in 60 patients with liver transplantation, about 5.7 % of them had a tumor recurrence in explants within 40 months [183]; and in 7 out of 22 patients, their donor liver transplantation were characterized with microvascular invasion within 3 years [55]. These data indicate that CTCs can self-seed and grow preferentially at regions or in the vicinity from their primary tumor. These sites may represent specific metastatic niches for a CTC where, presumably, stromal cell types, extracellular matrix proteins, and diffusible signals that support the survival and self-renewal of reseeded CTCs. Thus, it is required to further explore the mechanisms for reseeding by CTCs after tumor resection or orthotopic liver transplantation, thereby promoting the development of new strategies for the prediction and prevention of postoperative recurrence.

12.2.6 Heterogeneity of CTCs

Cancer is a heterogeneous disease. The same tumor mass can contain genetically distinct cell populations with independent tumor propagating capability, but significantly different phenotypic and functional characteristics [43]. There is now increasing studies that demonstrated a significant discrepancy in molecular expression between primary tumor and

corresponding distant metastatic sites, as well as among multiple metastatic sites [131, 166, 181]. This heterogeneity might be explained by genomic instability in cancer and selective pressure against different tumor cell clones under various systemic therapies [85]. The genotypic variation between primary cancer and CTCs was also demonstrated [40, 85, 166, 199]. Additionally, emerging evidence exists regarding high heterogeneity of CTCs, even within one histological distinct tumor type, as well as within one patient [13, 40, 61, 85, 148, 166, 199]. Recent studies have shown that the markers in CTCs may also change over the course of therapy [59, 74, 148, 189]. Single cell analysis may further reveal tremendous cell-to-cell variability.

Biological and clinical relevance of CTC heterogeneity is still under investigation. At least, heterogeneity of CTCs could limit their metastatic potential in the blood circulation, for example, by "diluting" the effect of platelets [49, 160]. The latter are known to act as EMT inducers [102] and as protectors for immune-mediated lysis [9]. Technically, heterogeneity of CTCs represents a challenge for the development of CTC measurements. An ideal CTC assay would require the isolation of all types of CTCs without any loss, but heterogeneity of CTCs highlights the difficulty of using antibodies or cocktails of antibodies to capture and identify all types of CTCs due to a lack of specific markers expressed in all populations of CTCs and not in nontumor cells. In a word, as a biological characteristic of CTCs and a challenge for the development of CTC tests, heterogeneity of CTCs enforces the need for a broader range of markers in order to isolate and target the rare subset of CTCs with increased metastatic potential.

12.3 CTCs as a Liquid Biopsy

The development of personalized precision therapies for cancer patients depends on efficiently obtaining representative tumor specific information by the identification of the molecular drivers of their disease. Currently, tumor biopsy samples are most often used to identify biomarkers for prediction of therapy response, and usually obtained only at time of initial diagnosis. However, obtaining a tissue sample by image-guided needle biopsy or tumor excision is an invasive procedure with associated risks and distressing to subjecting patients, and certain locations (e.g., liver, lung or brain) are especially difficult to access and not always feasible. In addition, biopsies frequently yield poor quality and/or insufficient quantity, and therefore provide only limited information about the genetic content of cells. Owing to profound intratumor heterogeneity, single-site biopsies are unlikely to capture the complexity of the genomic landscape of a patient's tumors. Due to the presence of multiple metastatic foci or anatomically challenging, metastatic cancer samples are usually not available or readily obtainable in the clinic. Furthermore, cancer is continually evolving at the molecular level, or its genomes are unstable and prone to changes under selection pressures such as the application of therapies, whether a diagnostic biopsy sample originally from primary tumor truly represents the patient's disease status is questionable. Following tumor evolving or metastatic development, the treatment often continues to be based on molecular characterization of the primary tumor despite discordance between the primary and metastatic lesions or between various metastatic tumors in the same patients. So repeat biopsies are often needed for serial monitoring of tumor molecular profile status to ensure that a given targeted therapy is still "hitting the target" or to identify new predictive biomarkers for emerging secondary drug resistance and disease relapse. Collectively, the translation of laboratory findings to the clinical setting has been hampered by the inability to obtain adequate material for serial monitoring of tumor genotypes. Thus, there is a pressing need for a much less invasive and cost-effective alternative approach able to easily and serially access tumor tissue at various time points during a disease course. Since CTCs are released into the blood circulation from multiple sites of primary and metastatic lesions, and might likely better represent the overall heterogeneity of tumor than any single tumor biopsy at the time of necessary intervention, they are often referred to as a "liquid biopsy." As a minimally invasive alternative to tissue biopsy, the real-time tests of CTCs are not only reduced patient risk and lower costs, but also informative for serial monitoring of the genetic changes in any situation, particularly useful in cases where relapsed tumors are very different from the original primary ones, or in assessing liver, lung, or brain cancer where it is hard for an ordinary biopsy.

12.4 Potential Clinical Applications of CTCs

CTCs in the bloodstream play a critical role in establishing metastases. The ideas about their clinical applications have changed with time. Research on CTCs is now a very active field, and the clinical value of CTCs as a biomarker has been widely explored in recent year. Potential clinical utilities of CTCs may span from disease diagnostic biomarkers for early cancer detection, to prognostic biomarkers for monitoring therapy response, and predictive biomarkers for choosing therapeutic drugs.

12.4.1 Early Diagnosis for Subclinical Cancers

Cancer invasion and hematogenous spread can be an early event in the natural history of carcinogenesis, implicating that a primary tumor and a metastatic lesion could grow in parallel [48, 85]. Current screening methods are unable to detect this early spread, and small invasion lesions that are detectable in patients by current imaging procedures usually contain more than 10^9 tumor cells. The ultrasensitive diagnostic detection of CTCs could provide a tool for an early diagnosis of invasive cancers or undetectable metastatic relapse in subclinical stage before they become detectable by imaging, for demonstration of the presence of a malignancy, and for potential establishment of the tissue of origin. In fact, CTCs are frequently detected in early stage cancer patients. However, the enumeration of CTCs has not been clinically adopted as a screening or primary cancer diagnostic assay.

12.4.2 Assessing Individual Prognosis

CTCs have been proposed to assess individual prognosis of cancer patients, and then stratify the patients at risk to specific adjuvant therapies. Identification of a subset of CTCs with metastatic potentials would provide clinicians a more complete picture of their patient's high risk for relapse, amenable for making a decision about the most appropriate treatments for each patient. Currently, the assessment of CTC counts is included in more than 400 clinical trials registered at www.ClinicalTrials.gov, most focusing mainly on prognostic biomarkers for breast, melanoma, lung, rectal, colorectal, prostate, and some other types of cancer [3, 132]. A growing number of clinical studies show a close connection between elevated CTC counts and worse prognosis of patients with various types of cancer, suggesting that CTCs are either surrogates of metastatic activity or causally involved in the promotion of metastasis [14, 24, 109, 204].

12.4.3 Guiding the Individual Therapeutic Decisions

Since cancers caused by specific genetic mutations respond well to certain medications, molecular analyses of CTCs have great potential to provide predictive information on response to a specific therapy, and thereby determine which targeted drugs to give a patient. In case of primary resistance, initial therapy response should be assessed as soon as possible. Clearly, such analysis can be repeated to spot any new mutations, allowing an early detection of resistance, primary or secondary, to therapies and potentially driving the switch to another, more effective, targeted drug, or a more appropriate alternative treatment.

12.4.4 Monitoring Treatment Response

Due to changes in cancer biology and response to treatment over time, longitudinal predicting and monitoring therapy response and disease progression is crucial for cancer management. Currently, imaging modalities, e.g., computer tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) or combinations are routinely done to monitor therapy response. Unfortunately, these modalities in general are not suited for detection of early therapy response due to lack of sensitivity. For targeted therapy, a reduction in tumor size associated with tumor response is not necessarily measurable, and in addition, an associated active inflammatory response may hide tumor response [57]. As the patient's blood contains a sufficient number of CTCs, the enumeration has been shown to enable monitoring of therapy response by performing reliable statistics to analyze an increase or a reduction in CTC counts [100]. Sequential measurements of CTCs at multiple time points along a patient's cancer journey may enable analyzing dynamic changes in CTCs and determining the treatment response or resistance during the course of therapy. As cancer can develop resistance against a given therapy and may then recur or spread, molecular characterization of CTCs beyond counting such as the timely identification of secondary mutations for biologic determinants of emerging drug resistance is of utmost importance allowing for pursuing more active agents for continuing therapy.

12.5 Current Technologies for the Isolation and Detection of CTCs

12.5.1 An Ideal Platform for CTCs and its Challenges

Over the past few years, there has been a lot of interest in the field of CTCs with rapid growth of developing new biotechnologies to identify CTCs. Ideally, a typical platform for isolating and detecting CTCs should have the following characteristics [13]:

(1) high sensitivity (or recovery rate): ability to isolate or detect the smallest number of CTCs per sample. This is particularly important for the early diagnosis of the primary tumor and metastasis with low number of CTCs in the peripheral blood; (2) high specificity (or purity rate): no false positives; (3) reproducibility: an acceptable intra-operator or inter-operator variability; (4) great purity: high

ratio of isolated or detected CTCs to all detected or isolated cells from a sample; (5) intactness and viability: high-purity isolation of viable CTCs is very important for propagation of CTCs and exploitation of their potential, such as drug efficacy test in culture or in xenograft models; (6) high throughput: the sample is processed in a relatively short period of time; (7) low cost for patients.

However, two main factors make the isolation and detection of CTCs formidable technical and analytical challenges [13, 76]:

(1) CTCs are extremely rare in the peripheral blood of cancer patients relative to hematological cells, typically comprising only a few CTCs per milliliter of whole blood, which contains $\sim 7 \times 10^6$ white blood cells, $\sim 5 \times 10^9$ red blood cells, and $\sim 3 \times 10^8$ platelets. Mature erythrocytes can be easily removed from blood since they have distinct biological, chemical, and physical properties. However, leukocytes and CTCs share many common properties, so effective separation of CTCs from leukocytes is quite difficulty.
(2) There is no one marker that can reliably and efficiently distinguish these CTCs from other bloodborne cells. CTCs do not universally express specific tumor markers. Moreover, CTCs are inherently heterogeneous.

12.5.2 Enrichment Methods

Due to their rarity, most CTC isolation and detection techniques are preceded by an initial enrichment step. The enrichment step typically removes the majority of unwanted hematopoietic cells from the sample and improves the relative concentration of CTCs. The enrichment includes a large panel of technologies based on the different biochemical and biophysical properties of CTCs compared with normal hematopoietic cells. Biochemical properties generally involve surface biochemical antigens of cells, allowing for marker-labeling separation. Biophysical properties involve cell size, density, shape, electrical polarizability, deformability, viscosity, or stiffness (associated with a different cytoskeletal structure of cancer cells), allowing for biophysical label-free separation that may avoid the problem of the epithelial antigen bias by existing biochemical methods. Furthermore, due to no modification, cells isolated using physical separation processes can be used for a wider range of analyses, especially those requiring viable cells. Common methods for sample enrichment include density gradient centrifugation, red blood cell lysis, size-based filtration, and positive or negative immunomagnetic separation.

12.5.2.1 Density Gradient Centrifugation

Based on the particular lower density of the tumor cells, epithelial cells, platelets, and low-density leukocytes, these cells of similar density can be separated from other leukocytes and erythrocytes by using a density gradient medium. Density gradient centrifugation is now used widely as an enrichment step prior to detection method. To generate density gradients, layer after layer of gradient media is placed in a tube with the heaviest layer at the bottom and the lightest at the top in either a discontinuous or continuous mode. The cell fraction to be separated is placed on top of the layer and then centrifuged. Common issues by this method are the mixing of blood with the gradient medium and the entrapment of CTCs within red blood cells (RBCs) during the procedure, both of which may result in nonspecific loss of desired cells. Many efforts have been made to improve the efficacy of the enrichment. For example, by placing a porous membrane on top of the gradient media to prevent the mixing, the issue of the mixing of blood with the gradient medium has been partly addressed (e.g., OncoQuick®, Greiner BioOne, Frichenhausen, Germany). Another variation of this method is enabled by RosetteSep™ (STEMCELL Tech., BC, Canada), which uses a mixture of antibodies specifically crosslinking RBCs to each other and to white blood cells (WBCs), resulting in the formation of cell rosettes consisting of multiple RBCs and WBCs and effective separation of CTCs from the higher density of these clusters [215].

12.5.2.2 Microfiltration

Microfiltration methods permit smaller hematopoietic cells to pass through pores of varying geometries but retain larger CTCs. The microfilters can be categorized into three basic types of structures: weir structures consisting of microchannels with a sudden decrease in the channel cross section, pillar structures consisting of an array of microposts spaced appropriately to form constrictions to capture target cells, and pore structures consisting of a membrane perforated with a two-dimensional (2D) or three-dimensional (3D) array of small holes. The fluid flow rate and the cross-sectional opening of the constrictions are the key design parameters in these microfilters. The combination of the two determines the threshold size, shape, and deformability of target cells that can be captured by the filter [96]. Isolation by size of epithelial tumor cells (ISET) (RareCells, Paris, France) through a polycarbonate membrane with calibrated pores 8 μm in diameter was developed as an early filter for the isolation of tumor cells [186]. Currently, several commercial microfilters with pore structures have been introduced, including the ClearCell®, Rarecells®, and ScreenCell® devices. However, since cell size varies considerably not just

between different types of cancer, but also between different cells of the same cancer, small CTCs can be lost during this process. In particular, EMT-related CTCs may not be stiffer than leukocytes and might therefore not be retained by these filters. Furthermore, these microfilters have the potential for clogging when large numbers of cells are processed, which can cause unpredictable variations in flow rate and consequently in the force applied to squeeze cells through each constriction. Another problem of filtration is a possible adsorption of cells onto the filter membrane, which leads to difficultly detach for further analysis.

12.5.2.3 Immunological Enrichment

Immunological enrichment is usually achieved by magnetic activated cell sorting (MACS), a method depending on antibody-based capture with magnetically labeled antibodies. MACS can be used for positive or negative enrichment with the antibodies targeted against cell surface markers of tumor cells or hematopoietic cells.

12.5.2.4 Positive Selection

Positive selection captures target cells by using antibodies that bind to the surface of cells expressing specific antigens. Among cell surface markers for positive selection, EpCAM has been widely applicable to various different cancer types [13, 76]. With immunomagnetically labeled EpCAM antibodies, CTCs expressing EpCAM are positively enriched from whole blood. Several techniques have been commercially applied for positive enrichment, such as MagSweeper [170] and AdnaTest (AdnaGen AG, Laggenhagen, Germany) [10]. The CellSearch system (Veridex, LLC, Warren, NJ, USA), an available technology approved by the US Food and Drug Administration (FDA) for use in a clinical setting, also immunomagnetically enriches cells expressing EpCAM [118]. To circumvent the limitation that rare CTCs are detected in a small amount of blood, GILUPI GmbH has designed an EpCAM-coated wire (CellCollector™) to capture CTCs directly in vivo [154]. This medical wire is positioned through a cannula into the peripheral arm vein of a cancer patient. It is estimated that for a duration of 30 min, up to 1.5 L of blood flows over the detector, thus increasing the yield of detectable CTCs. However, isolation of CTCs based on positive enrichment is limited to the targets chosen. Using EpCAM as a target, only cells expressing EpCAM are isolated, and CTCs that have undergone EMT and no longer express EpCAM or expressing non-epithelial phenotypes are not captured. Alternatively, negative enrichment targeting hematopoietic cells could deplete all cells from blood samples, except CTCs, probably combating these obstacles.

12.5.2.5 Negative Enrichment

Negative selection is highly advisable as an unbiased enrichment step prior to the detection analysis since it has several other advantages including potential time/cost-efficiency and improved sample yield and purity [13, 76, 211]. Depletion of CD45(+) leukocytes is a preferred approach widely used to enrich CTCs lacking adequate EpCAM expression. Using this approach, viable tumor cells have been cultured [13, 131]. To improve the yield and to displace unwanted erythrocytes, CD45 depletion is usually combined with other label-independent methodologies such as density gradient centrifugation or red blood cell lysis [76, 211]. However, both methods may lead to false-negative results due to a loss of tumor cells. Another example of negative enrichment is the RosseteSep (StemCell Technologies, Vancouver, BC, Canada), which uses a mixture of antibodies specifically targeted against hematopoietic cells and crosslinks unwanted cells in whole blood to multiple RBCs and WBCs, forming cell rosettes and effective separation of CTCs from the higher density of these clusters. When centrifuged over a buoyant density medium such as Ficoll-Paque, the formation of immunorosettes increases the density of the unwanted cells and causes them to pellet along with the free RBCs [42, 114]. The depletion procedure probably leads to non-specific cell loss, and needs to be performed carefully.

12.5.3 Detection Methods

Following enrichment, CTCs can be detected and molecularly characterized by using nucleic acid or cytometric techniques.

12.5.3.1 Nucleic Acid-Based Molecular Detection

Many genomic techniques could be applied for analysis of genetic alternation in isolated CTCs focusing either on DNA level or on mRNA level, such as gene microarray for gene expression profiling; comparative genomic hybridization (CGH) or array CGH (aCGH) for whole genome screenings for chromosomal gains at losses; fluorescence in situ hybridization (FISH) for the presence or absence of specific DNA sequences on chromosomes, known gene amplifications, mutations, deletion, copy number alterations, or chromosomal abnormalities; single nucleotide polymorphisms (SNP) chip for SNP combination patterns; microRNA array for microRNA expression profiles; and methylation array for genome-wide DNA methylation mapping. Among them, FISH is currently proposed as a valid method for further genotyping of isolated CTCs although it is labor-intensive, and requires a high skill level [54, 92, 111, 130, 169].

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) can sensitively quantified nucleic acids and has been commonly used to identify CTCs by analysis of cell- or tissue-specific mRNA makers. The specificity of

these assays for CTCs depends on the assumption that normal tissue cells do not circulate, unless they become tumorous. The mRNA markers usually used for the identification of CTCs from peripheral blood mononuclear cells are the tumor-associated, organ-specific, or epithelial-specific markers, such as *cytokeratins* (*CKs*) *19* and *20* for colorectal cancer, *prostate-specific antigen* (*PSA*) for prostate cancer, *telomerase reverse transcriptase* (*TERT*) for gastric cancer, *MUC-1* and *human epidermal growth factor receptor-2* (*HER2*) for breast cancer, *carcinoembryonic antigen* (*CEA*) for colorectal cancer, and *alpha-fetoprotein* (*AFP*) for primary liver cancer [76, 136, 211]. However, due to its high sensitivity, RT-PCR may not be suitable for analyzing CTCs in cell preparations with a high number of contaminating non-target cells. Normal blood cells also express many transcripts at low levels, so PCR-based assays are required to validate cut-off values to overcome the problem of false positives. Moreover, target transcripts might be downregulated in CTCs, multiplex PCR targeting the numerous tumor-associated mRNA transcripts favor overcoming the problem of false negatives. Another limitation is that once the cell is lysis and no longer viable, further cell-based analysis such as enumeration, cell culture, or sensitivity assays could not be performed.

12.5.3.2 Protein-Based Molecular Detection

Currently, the expression of epithelial markers (EpCAM, CKs) and the absence of the leukocyte marker CD45 are widely accepted as definition of CTCs. It has been applied to the CellSearch system for enumeration of CTCs in blood of patients with metastatic breast, colorectal, and prostate cancers [118]. The captured CTCs are stained with a combination of anti-CK8/18/19, anti-CD45 fluorescently conjugated dyes, and the nuclear dye 4′,6-diamidino-2-phenylindole (DAPI). Based on positive staining for CKs and DAPI, negative staining for CD45, cell size, shape, and the nucleus-plasma relation that might be disturbed by atypical enlarged nuclei in CTCs, enumeration of rare CTCs is accomplished by immunofluorescence [13, 118, 124]. In addition, CTCs can also be defined based on the expression of mesenchymal markers (e.g., vimentin), or stem cell markers (e.g., CD133, CD44) [103, 139, 141]. Since CTCs are detected by using immunofluorescence, enrichment and purity only need to be good enough for the output cells to be imaged and identified in a reasonable amount of time. However, blood samples may contaminate epithelial cells, and CTCs have undergone EMT and no longer express CKs, CK-based immunocytochemistry methods therefore carry a risk of false positive and false negative results.

12.5.3.3 Flow Cytometry

Another widely used cytometric technique for CTC detection is flow cytometry, which is based on fluorescence-activated cell sorting (FACS). Because multiple parameters can be simultaneously measured, flow cytometry separates a specific population of cells with high purity. A limitation to flow cytometry is throughput; since each cell must be sorted individually, limited amount of cells can be analyzed. To address this issue, in vivo flow cytometry has been investigated to detect and enumerate CTCs directly in the bloodstream of murine cancer models [52, 62, 126], or to monitor the dynamics of CTCs continuously. Since the entire blood volume is potentially used as the specimen, such methods have higher detection sensitivity. Fan et al. [52] combined in vivo flow cytometry with a green fluorescent protein (GFP)-transfected HCC orthotopic metastatic tumor model for real-time monitoring of CTC dynamics. However, efficient in vivo labeling of CTCs and potentially toxicity of fluorescence used to label cells to human body remain the main challenges in this approach.

12.5.4 Function-Based Detection

Function-based assays potentially specifically detect viable CTCs and secreted proteins by CTCs. The epithelial immunospot (EPISPOT) assay (University of Mountpellier, Mountpellier, France), an adaptation of the enzyme-linked immunospot assay (ELISPOT), is a functional cell culture assay, allowing for counting CTCs by observation of immunospots resulted from enough secreted specific marker proteins (CKs, MUC1, PSA, etc.) after short-term culture [4]. Another functional detection method is the collagen adhesion matrix (CAM) assay. Based on the ability of tumor cells to digest and invade into the connective tissue-like material, the assay allows for a function separation of more invasive and aggressive type of CTCs [105]. In this method, whole blood is incubated in a special blood collection tube, and CTCs adhere to the internal coating of CAM. After non-adhered cells are washed away, CAM is then broken down by collagenase treatment to release CTCs. TelomeScan (Oncolys BioPharma Inc., Tokyo, Japan), a telomerase-specific replication-selective adenovirus expressing green fluorescent protein, is used to infect and visually detect live CTCs among millions of peripheral blood leukocytes [87]. The specificity is achieved by making adenovirus replication only possible in the presence of telomerase activity, which has been identified as a relevant marker of cancer cells. These techniques allow a functional separation of viable, protein-excreting, more aggressive CTCs without relying on epithelial markers. In theory, viable cells have more clinical relevance than apoptotic cells as they should still be capable of forming metastases. Furthermore, these new techniques may be especially used for drug development. However, these function-based assays could not detect some CTCs

because they may not survive during the transition from the bloodstream to in vitro culture.

12.5.5 Combined Enrichment and Detection of CTCs

In addition to the above-mentioned ISET, AdnaTest, CellSearch system, and EPISPOT, current assays combining both enrichment and detection also include the Ariol system (Leica Micorsystems, Bufalo Grove, IL, USA) and the CTC-Chip. The Ariol system combines both image capture and quantitative panel analysis [45]. Whole blood is first lysed to remove RBCs, and the enriched cells are then subjected to immunocytochemistry using the same detection approach as the CellSearch system. CTC-Chips are based on a unique microfluidic device with antibody-coated microposts, which allow the mixing of blood cells through the generation of microvortices to significantly increase the number of target cell-surface interactions with the antibody-coated chip surface under precisely controlled laminar flow conditions [45, 60, 153]. Such an approach enables selective and efficient capture of viable CTCs in a single step from whole blood samples without the need for an initial enrichment step. Several EpCAM-based microfluidic chips have been tested for highly efficient capture of CTCs. Furthermore, a number of versatile label-free microfluidic biochips coupled with pinched flow dynamics have been developed to effectively separate CTCs from both epithelial and non-epithelial cancers via their distinctively different physical properties such as deformability and size. Another novel CTC-iChip combines a size-based filtration with an affinity-based enrichment strategy by a series of the tumor membrane epitope-dependent or independent steps, thus enhancing the chance of systematic removal of peripheral blood mononuclear cells (PBMCs) and RBCs, and being applicable to isolate both EpCAM(+) and EpCAM (−) CTCs virtually in all epithelial and non-epithelial cancers [81, 129]. To date, however, many devices have been tested only with simple, low complexity samples.

12.5.6 Drawbacks of Current Detection Methods

Although many analytical techniques and approaches for isolation, detection, and characterization of CTCs have been developed in the past decades, no ideal method is currently available. Each individual methodology has its own advantages and disadvantages. By combining two methods, the disadvantages can only be partially overcome. In spite of a number of improvements, there are still many limitations for the standardization, automation, quality control, and accreditation of analytical methods that hinder the use of CTCs in the clinical setting. Different isolation and detection approaches may result in a substantial variability in the rates of positive samples, even in the same patient [64, 182]. Since most current methods of CTCs, such as EpCAM-based isolation methods and CK-based detection methods, are based on epithelial markers, they cannot detect CTCs with EMT phenotype and likely miss some of aggressive CTCs with a non-epithelial phenotype. This subtype of cells probably has experienced EMT and is more capable of causing metastasis [13, 131, 211]. The bias against such tumor cells can be corrected by using a broad-spectrum specific cocktail of epithelial and mesenchymal markers on cell surface covering all potential phenotypes of CTCs. This cocktail, however, would lead to false-positive results because that could increase the chance for cross-reaction of some of these markers with blood cells and/or other circulating non-tumor cells [78, 108]. Additionally, CTCs from a single blood sample harbor different genetic aberrations [61, 148], thus molecular characterization of CTCs could provide clinicians with more information beyond simple enumeration. Moreover, most of current separation methods do not keep cells viable. Therefore, we can see a great need to develop more gentle and comprehensive techniques for detecting all subtypes of CTCs and keeping cells viable to be used for determination of their metastatic potential. Finally, in order to evaluate their quality and validity, all the analytical methodologies should be validated in appropriately sized clinical trials.

12.6 Other Emerging Technologies for the Analysis of CTCs

12.6.1 Single Cell Analysis

Advances in single-cell molecular analysis will enhance our ability to explore mechanisms of metastasis. Sequencing technology is a powerful tool for the analysis of specific genomic aberrations, especially in the setting of cancer. With regards to CTC analysis, sequencing tends to be applied more frequently at the level of RNA; however, several studies have also interrogated CTCs at the DNA level [10, 74, 118, 154]. In general, for processing at the RNA level, total RNA or mRNA is extracted from CTCs following enrichment. Isolated RNA is then reverse transcribed into cDNA and PCR amplified using primers that are specific to the mutant/target region. Amplified mutations can be detected using either gel electrophoresis for known length transcripts, and/or analyzed with one of the several commercially available sequencing platforms. For processing at the DNA level, instead, total DNA is extracted from CTCs, whole genome amplified using

commercially available kits, and subsequently amplified via PCR using specific primers.

Sequencing techniques also have several marked disadvantages including: (1) limitations with regards to sensitivity that make single cell analysis difficult, with many groups reporting the need for a minimum of 50 or more CTCs for adequate results [74, 114]; and (2) leukocyte contamination and the inability to visually confirm the source of amplified transcripts can lead to false positive/negative results. However, several groups have attempted to utilize single cell micromanipulation (selecting for CTCs based on immunofluorescent staining prior to the collection of DNA/RNA) [10] and/or adapted PCR protocols (e.g., nested PCR) [74] to combat these issues with promising results.

12.6.2 Culture of CTCs

Characterization of fixed CTCs provides relatively little data about their metastatic potential and functional capability. The option to isolate viable CTCs for genotyping and functional characterization is a tremendous potential of CTCs. Therefore, after isolation of CTCs by the different methods, culture of CTCs is required for functional studies such as drug sensitivity or resistance test, and for the propagation of a larger pool of cells allowing for the performance of genomic and correlative work. Another intriguing possibility is the ex vivo manipulation of CTCs for cellular therapy of cancer. Theoretically, if enough CTCs could be expanded, we could use them to develop personalized cancer immunotherapy. To this end, improved methods including specific culture media and appropriate culturing conditions for the growth of unmanipulated CTCs, but not for the other epithelial or non-epithelial cells, have to be developed through experiments. However, culture of CTCs is a much more complex procedure than culture of primary tumor cells from a primary tumor or from another cell population, and mimicry of the tumor microenvironment in vitro is particularly difficult. In addition to a very limited number, CTCs are not relatively protected from cell death and the harsh environment and shear stresses of the vascular circulation, and can lose their derive and original markers. So the development of technologies for CTC culture is highly challenging and extremely promising.

Currently, primary CTCs isolated from blood samples of cancer patients on different platforms are expandable in vitro [36, 71, 135]. Paris et al. [135] have shown that isolated CTCs from patients with prostate cancer can be expanded in culture for up to 14 days. Similar work has been conducted with CTCs isolated from lung cancer, breast cancer, and urinary bladder cancer patients [36, 71].

Recently, several studies have introduced new methods for in vitro cultivation of CTCs from patients with various types of cancer. In a proof-of-concept study [36, 71, 207], CTCs from patients with metastatic breast cancer proliferated best as tumor spheres when cultured in serum-free media supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) under hypoxic conditions. The proliferation of cultured CTCs as non-adherent spheres appeared to be critical for establishment of long-term oligoclonal CTC cultures, which could be sustained in vitro for more than 6 months, and were tumorigenic in mice. Cayrefourcq et al. [35] have established a permanent cell line from CTCs of one patient with colon cancer. This cell line induces in vivo tumors after xenografting in immunodeficient mice, resembles characteristics of the original tumor cells in the patient with colon cancer, and displays a stable phenotype characterized by an intermediate epithelial/mesenchymal phenotype, stem cell-like properties, and an osteomimetic signature, indicating a bone marrow origin. As for HCC, we recently used a 3D cancer model (spheroid formation on Matrigel culture) for drug evaluation to assess the sensitivity of isolated HCC CTCs to sorafenib, a multitargeted, small-molecule tyrosine kinase inhibitor approved for the treatment of advanced HCC [94].

Cancer cell therapy has a promising future, which is largely dependent on tumor antigens. Another intriguing possibility is the ex vivo manipulation of CTCs for cellular therapy of cancer. The rate-limiting step in this approach is the low yield associated with many CTC platforms. In theory, if enough CTCs could be obtained and expanded, they could be used as a platform for the development of personalized tumor immunotherapy.

12.6.3 Experimental Models for CTCs

Over decades, different mouse tumor models have been developed. CTCs were looked for in some of these animal models. Taking HCC for an example, an experimental model of human orthotopic HCC transplantation in immunodeficient mice allows the continuous generation of CTCs [155, 156]. During tumor development, tumor cell spreading is an early event. The number of CTCs was correlated with the tumor size, and decreased dramatically after resection of the tumor [47, 180]. Similar work has been performed by Fan et al. [52]. In the orthotopic HCC model, CTC dynamics were correlated with tumor growth, the number and size of distant metastases correspond to CTC dynamics, and the number of CTCs and early metastases decreased significantly after the resection.

However, these studies used cell lines to develop mouse tumor models, and may not entirely unravel some aspects of

CTC biology. Alternatively, xenotransplantation of primary CTCs into immunodeficient mice is an active area of research, because the use of xenograft models may gain further insights into the biology of CTC release and may test responses to newly designed therapies. In small cell lung cancer, captured CTCs from blood of patients grew into tumors after transplanted subcutaneously into immune-compromised mice. These tumors histologically resemble the primary malignancy, and mirror the donor patient's response to chemotherapy [65]. In a study by Yang et al. [205, 206], CD90(+)CD45(−) cells from blood samples of HCC patients generated tumor nodules in immunodeficient mice. Serial transplantation of CD90(+) cells from tumor xenografts generated tumor nodules in a second and subsequently a third batch of immunodeficient mice. In another study by Sun et al. [168], after three months following xenotransplantation, 50 % of the mice injected with EpCAM(−)CD45(−) cells isolated from blood of HCC patients developed subcutaneous nodules, while none developed from EpCAM(−)CD45(−) cells.

12.7 Advances in Detection and Molecular Characterization of CTCs in HCC Patients

12.7.1 Detection of CTCs in HCC Patients

It was initially suspected that recurrence and metastasis following the treatment of HCC was caused by incomplete surgical resection, and therefore expanded radical resections were employed. However, this approach was generally unsuccessful, leading to new hypotheses that either the intrahepatic dissemination of tumor cells through the portal vein branches or de novo tumorigenesis are the cause. Yet, these hypotheses fail to explain why early tumor recurrences commonly occur at the site of transplanted allograft in cases of liver transplantation [115], and thus CTCs as an alternative source of tumorigenic tissue were proposed.

By using PCR-based method for detection of liver-specific or tumor-associated gene expressions in peripheral blood mononuclear cells, HCC CTCs have been demonstrated to be present in circulating peripheral blood. These gene mRNA markers include *AFP*, *albumin*, *TERT*, *Snail*, and *insulin-like growth factor-binding protein 1* (*IGFBP1*) [119, 128, 136, 187]. Among them, only AFP is a well-established HCC marker [210]. However, AFP is not a HCC-specific marker, and a large percentage of HCC cases are negative for AFP [210]. Microfilters were also used for CTC detection in patients with HCC [186], and enabled visualization and counting of liver-derived tumor cells and microemboli. By using the ISET technique, for example, Vona et al. [186] identified ≥ 1 CTCs/7.5 mL blood in 23 of 44 (52 %) patients, with a range of 3–33 CTCs/7.5 mL blood, and Morris et al. [122] also identified ≥ 1 CTC/7.5 mL blood in 19 of 19 (100 %) samples, with a range of 13–158 CTCs/7.5 mL blood.

As mentioned, EpCAM-based methods have been widely applicable to detection of CTCs in various different cancer types. Robust evidence revealed that the patterns of EpCAM expression in the liver are different from that in other epithelial organs although the liver is also an epithelial organ, and contains two major differentiated cell types: the hepatocyte and the bile ductule cell. In the embryonic liver, EpCAM is expressed in the majority of hepatocytes, while in the adult liver, EpCAM is expressed only in bile duct epithelium, but not in hepatocytes [44, 157]. In the cirrhotic liver, EpCAM is expressed in proliferating bile ductules derived from hepatic progenitor cells [44]. EpCAM is also expressed in both hepatic stem cells and fetal hepatoblast cells [157]. In liver neoplasia, almost all cholangiocarcinomas express EpCAM, whereas only a small percentage of HCC cases are positive for EpCAM [44, 143, 144, 194]. These observations definitely suggest that EpCAM-based strategies, including the CellSearch system, will miss most HCC CTCs. Consistent with these results, indeed, the low number of CTCs was generally detected in patients with HCC using the CellSearch system. For instance, in 7.5 mL blood, Sun et al. [168] detected ≥ 2 CTCs (range, 1–34 CTCs) in 41 % (51/123) preoperative HCC patients, Schulze et al. [158] detected $\geq$ 1 CTC (range, 1–5 CTCs) in 31 % (18/59) patients with HCC across a range of disease stages, and Morris et al. [122] detected $\geq$ 1 CTC in 28 % (14/50) patients (range, 1–8 CTCs). In contrast, a parallel test using the ISET technique conducted by Morris et al. detected $\geq$ 1 CTC in 100 % (19/19) samples (range, 13–158 CTCs), indicating as anticipated poor concordance between EpCAM (+) CTCs and HCC CTCs. Collectively, EpCAM-based strategies are not unsuitable for detection of CTCs in HCC patients, and it is required to develop a special method to capture CTCs in HCC patients.

It has been known that the asialoglycoprotein receptor (ASGPR) is an abundant transmembrane receptor exclusively expressed on the surface of hepatocytes, and can recognizes and internalize glycoproteins that have exposed terminal galactose and N-acetylgalactosamine residues [11, 164]. By using glycosylated macromolecules as vehicles to be selectively recognized by ASGPR, liver-targeting systems for genes and drugs have been developed [138, 172, 188, 190]. Therefore, we developed a unique method to magnetically separate CTCs in HCC patients, mediated by the interaction of the ASGPR with its ligand or antibody [93, 200]. Briefly, following an initial step of density gradient centrifugation for whole blood, enriched HCC cells were captured by biotinylated asialofetuin, an ASGPR ligand, and subsequently

labeled by anti-biotin antibody-coated magnetic beads (a ligand-receptor binding assay) or captured by ASGPR antibody (an antibody-antigen binding assay). The isolated HCC cells by magnetic separation were then identified by immunofluorescence staining by using the hepatocyte-specific antibody Hep Par 1 or CKs or their combination. Given the generally accepted absence of normal hepatocytes in circulation, blood cells labeled with the ASGPR ligand or antibody are thus considered to be circulating HCC cells. The ligand-receptor binding assay has an advantage that captured CTCs are alive and could be readily released with ethylene diamine tetraacetic acid (EDTA) from biotinylated ligand, allowing their culture for functional studies or single cell sequence. However, it has its own limitations: (1) Because the reaction is dependent on calcium ions, only heparin but not EDTA and sodium citrate can be used as anticoagulants for the blood sample. Addition of heparin was reported to cause gelling of cell suspensions in the purification of lymphocytes [7]. During the process of magnetic separation, due to gel formation, mononuclear cell suspensions from whole blood in heparin may slowly flow over a magnetic separator, probably affecting CTC separation efficiency. (2) The CTCs must be alive for the ligand-receptor binding assay, which requires more rigorous methods to properly collect, preserve, transport, and process specimens. (3) Various microenvironmental factors may change cell surface receptor activity. For example, calcium could induce a conformational change in the ligand binding domain of the receptor, and pH may regulate receptor function by altering the amount of calcium bound to the receptor [46, 197, 214]. Alternatively, these disadvantages could be avoided or reduced by the antibody–antigen binding assay.

We detected CTCs in blood samples from 85 HCC patients at various clinical stages by using the ligand-receptor binding-based approach [200]. CTCs were detected in 69 of 85 (81 %) HCC patients, even in those at early stage or with a tumor size < 2 cm. The number of CTCs detected ranged 0–125/5 mL, with an average of 19 ± 24 (mean ± SD). Either the positivity rate or the number of CTCs in patients with portal vein tumor thrombus (PVTT) was higher than that in patients without PVTT ($Ps < 0.001$), suggesting PVTT as an active source of systemic spread of CTCs. Furthermore, either the positivity rate or the number of CTCs was highly correlated with tumor-node-metastasis (TNM) staging, ranging from 66 % in stage I to 100 % in stage IV ($P = 0.003$), and from 3 ± 4 in stage I to 67 ± 35 in stage IV ($P < 0.001$), respectively. Both the positivity rate and the number of CTCs in patients beyond Milan criteria were higher than those in patients with in Milan criteria ($Ps < 0.01$), a most widely used criteria for the selection of candidates for liver transplantation [113], suggesting that detection of CTCs may have potential applications in selecting HCC patients for liver transplantation. Finally, both the positivity rate and the number of CTCs were also correlated with Edmondson-Steiner grading ($Ps < 0.05$), an authorized and extensively used histological classification [213]. Later, Li et al. [95] used the same method to detect CTCs in the peripheral blood obtained from 60 HCC patients, and confirmed our above-mentioned results. Furthermore, 31 HCC patients who received resection of liver cancer or hepatic artery chemoembolization were followed up for a minimum of 1 year, and the patients with CTC positive had a significantly higher rate of recurrence or metastasis (88.0 %) and a significantly higher rate of mortality (64.0 %) than the patients with CTC negative (16.7 %, $P = 0.002$; and 0.0 %, $P = 0.007$). Therefore, the authors suggest that CTCs may be used as a valid indicator to evaluate the progression and prognosis of HCC.

However, ASGPR-based capture strategy does not detect HCC CTCs that lack expression of ASGPR because the expression of ASGPR is heterogeneous in human HCC, and not all HCC tissues and human liver cancer cell lines express ASGPR [73, 161, 179]. We recently improved the detection method of HCC CTCs, which involves an initial depletion of CD45(+) leukocytes from the sample, followed by detection of CTCs with a combination of two antibodies against liver-specific markers, ASGPR, and carbamoyl phosphate synthetase 1 (CPS1) (Fig. 12.2) [99].

CPS1 is a mitochondrial urea cycle enzyme, a newly identified protein for Hep Par 1 [32]. Due to its detection only in hepatocytes, Hep Par 1 stain is commonly used as definitive proof of the hepatocellular origin of neoplasms in diagnostic surgical pathology. However, heterogeneous expression of CPS1 was also found in human HCC [93, 97, 99, 162, 175, 188, 190]. For example, Timek et al. [175] reported that 17/18 small tissue biopsy specimens of HCC were positive for Hep Par 1, but only 19/29 fine-needle aspiration biopsy specimens were positive. To increase detection of cells that may express only one of the two markers, we used an antibody cocktail against liver-specific antigens (ASGPR and CPS1). This method was specific for HCC CTCs, since other types of cancer cells such as breast cancer cells were not detected. The results showed that the improved system detected a higher count of CTCs in almost all patients examined than did the previous system [99], indicating that our previous methods underestimated HCC CTC population. Collectively, negative depletion enrichment combined with identification using a mixture of two antibodies against ASGPR and CPS1 not only increases sensitivity for CTC enrichment, but also provides high specificity for CTC detection in HCC patients, thereby minimizing false negative/positive results.

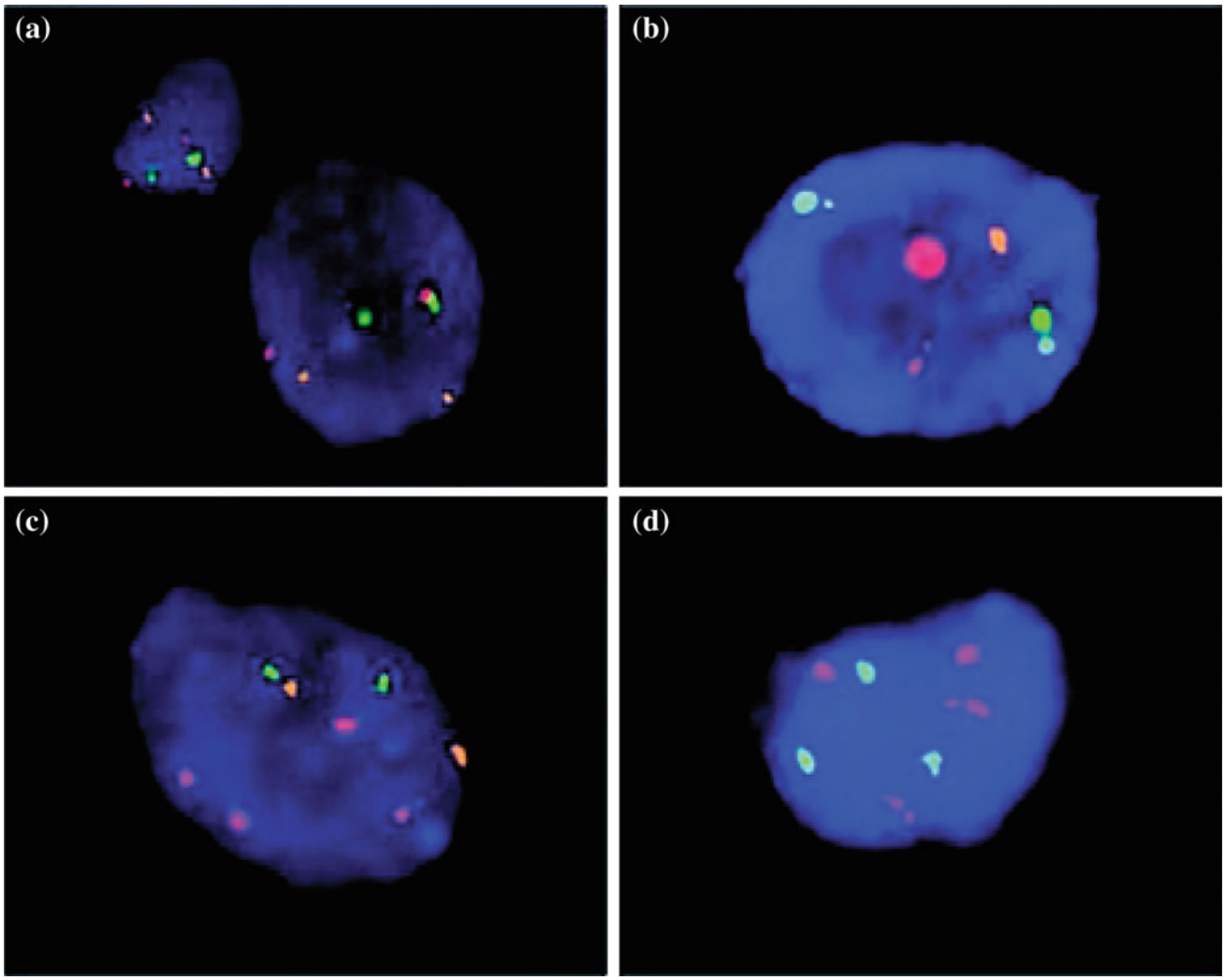

Fig. 12.2 Improved detection of circulating tumor cells (CTCs) from patients with hepatocellular carcinoma (HCC). Immunofluorescence staining of CTCs (*white arrow*) detected in blood from HCC patients with antibodies against asialoglycoprotein receptor (ASPGR) and/or carbamoyl phosphate synthetase 1 (CSP1) (*red*), and CD45 (*green*) with nuclear DAPI staining (*blue*) (*magnification* ×400). Reproduced from Liu et al. [99]. Permission from American Association for Cancer Research

12.7.2 Detection of So-Called "CCSCs" in HCC Patients

Accumulating research suggests that human cancers contain a small population of self-renewing cancer stem cells (CSCs) or CICs, which are proposed to be responsible for tumor origin, maintenance, and resistance to treatment. CSCs may disseminate from the primary tumor to distant sites through the circulatory system. Moreover, during EMT, CTCs seem to acquire more aggressive and stem-like traits, and have an increased ability to migrate into the bloodstream and display more resistance to anoikis [2, 208]. Thus, CTCs may contain a minor subset of CCSCs.

Over the past few years, several groups have made an effort to examine the existence of CCSCs in HCC patients by detecting CSC markers in CTCs, and assess their clinical relevance. For example, based on the suggestion that CD90 and CD44 are potential markers for CSCs, a research team detected circulating CD90(+)CD45(−) cells and circulating CD90(+)CD44(+)CD45(−) cells in HCC patients by using flow cytometry [51, 205, 206]. CD90(+)CD45(−) cells were detected in more than 90 % of blood samples from HCC patients but none from normal subjects or patients with cirrhosis [97, 205, 206]. The number of circulating CD90(+)CD45(−) cells was significantly positive related with that of CD90(+)CD45(−) cells [205, 206]. Later, the same group

conducted a prospective follow-up study [51] on 82 HCC patients who underwent partial hepatectomy for HCC, and whose blood was collected on the day before the operation. The results showed that after a median follow-up period of 13.2 months (range, 1.3–57.1 months), 41 patients (50 %) had recurrence. Compared to patients without recurrence, patients with recurrence had a higher median level of CCSCs (0.02 % vs. 0.01 %). CCSCs > 0.01 % could predict both intrahepatic and extrahepatic recurrence. Compared to patients with $\leq$0.01 % CCSCs, patients with > 0.01 % CCSCs had a lower two-year recurrence-free survival rate (22.7 % vs. 64.2 %) and overall survival rate (58.5 % vs. 94.1 %). On multivariable analysis, CCSCs > 0.01 %, tumor size, and tumor stage were all independent factors for prediction of recurrence-free survival. The authors concluded that CCSCs could accurately predict recurrence in posthepatectomy HCC patients [51]. Recently, Bahnassy et al. [16] also used flow cytometry to enumerate circulating CD90(+) cells, and suggested their important roles in the development and progression of hepatitis C virus (HCV)-associated HCC. Similar work on EpCAM(+) CTCs in HCC patients has been reported by Sun et al. [168].

As mentioned, EpCAM-based strategies are not unsuitable for detection of CTCs in HCC patients. Interestingly, EpCAM has been identified as a potential marker of liver CSCs [201], that stands in sharp contrast to that found in other types of the epithelial cancers [15], where CSCs or CTCs may downregulate EpCAM due to EMT [2, 23, 76, 131]. Consequently, EpCAM(+) HCC cells has been proposed to be tumor initiating cells with stem/progenitor cell features. Therefore, another approach to study of CCSCs in HCC patients is to detect EpCAM(+) CTCs in peripheral blood from HCC patients using the CellSearch system. Sun et al. [168] investigated the prognostic significance of EpCAM(+) CTCs in 123 HCC patients undergoing curative resection. CTCs in 7.5 mL of blood were present in 66.67 % of patients, with the cell counts ranged from 1–34. 51 patients had $\geq$2 CTCs preoperatively, and these patients developed tumor recurrence earlier than those with < 2 CTCs ($P < 0.001$). A preoperative CTC count of $\geq$ 2 was an independent prognostic factor for tumor recurrence ($P < 0.001$). On 1 month after resection, CTC-positive rates and CTC counts were significantly decreased (66.67 % vs. 28.15 % and 2.60 ± 0.43 vs. 1.00 ± 0.36, $Ps < 0.05$). Patients with consistent CTCs < 2 had lower recurrence rates than those with $\geq$ 2 CTCs (15.5 % vs. 87.50 %, $P < 0.001$). These results indicate that a preoperative CTC count of $\geq$ 2 is an independent predictor for recurrence in HCC patients after surgery. EpCAM(+) CTCs may serve as a real-time parameter for monitoring treatment response and a therapeutic target in HCC recurrence. The same group [58] later used qRT-PCR for detection of EpCAM mRNA in peripheral blood mononuclear cells from HCC patients, as determined by 76.7 % consistency with the CellSearch system, and further suggested prognostic significance of pretreatment CTC level in HCC patients treated with curative resection, transcatheter arterial chemoembolization (TACE), and radiotherapy ($Ps < 0.050$). Coincidentally, Huang et al. [69] recently demonstrated CTC counts measured by the CellSearch system as an important prognostic parameter for postoperative TACE on HCC recurrence.

Almost at the same time, Schulz et al. [158] also used the CellSearch system to investigate the prognostic relevance of EpCAM(+) CTCs in 59 patients with HCC. 18/59 (30.5 %) HCC patients had $\geq$ 1 CTC/7.5 mL, and 9/18 (50.0 %) CTC-positive HCC patients had > 1 CTC = 7.5 mL. The CTC-positive cohort had a significant shorter overall survival (460 vs. 746 days) ($P = 0.017$). At various Barcelona clinic liver cancer (BCLC) stages, CTC detection rates were significantly different: BCLC stages A 1/9, B 6/31, and C 11/19 ($P = 0.006$). 10/18 patients (55.6 %) with macroscopic vascular invasion and 10/16 patients (62.5 %) with microscopic vascular invasion exhibited CTC-positive findings ($P = 0.004$ and $P = 0.006$). These data also demonstrate frequent presence of EpCAM(+) CTCs in patients with advanced HCC and its prognostic value in terms of overall survival.

With the same purpose, Kelley et al. [83] recently used the CellSearch system to enumerate circulating EpCAM(+) epithelial cells in metastatic HCC patients. EpCAM(+) CTCs $\geq$ 2 were detected in 7/20 HCC (35 %), and CTCs $\geq$ 1 was significantly associated with high alpha-fetoprotein ($P = 0.008$) and the presence of vascular invasion ($P = 0.009$). Their findings corroborated the prognostic value of circulating EpCAM(+) CTCs in the previous studies [158, 168], supporting CTCs as a poor prognostic factor in metastatic HCC.

12.7.3 Molecular Characterization of CTCs in HCC Patients

Since CTCs are considered as direct triggers of cancer adaption, the current operative CTC definition becomes inadequate. Addition of stemness or EMT markers to criteria of CTC positivity seems to improve their biological relevance. To date, several studies have reported the feasibility of molecular profiling of isolated CTCs in HCC.

The Wnt/beta-catenin pathway is involved in the pathogenesis of HCC, and a significant subset of HCC has point mutations or deletions in the *beta-catenin* gene [140]. By DNA sequence analysis of the PCR product, Vona et al. [186] examined genetic mutations of *beta-catenin* exon 3 in 60 single CTCs and microemboli isolated from blood of 10 HCC patients, and found that two CTCs and one microemboli derived from three different patients had a *beta-catenin* mutation. These results suggested that cells carrying a *beta-*

catenin gene mutation were not specifically selected in CTCs spread from the primary liver tumor.

The functions of *TP53* gene, one of the most famous tumor suppressor genes, plays a central role in regulation of apoptosis and cell growth via mediating transcriptional activation of crucial growth regulatory gene. TP53 or its upstream/downstream pathways are frequently inactivated in almost all types of cancers, including HCC [26]. *HER2/neu* (*erbB-2*), one of the most famous oncogenes, encodes a receptor-like tyrosine kinase, p185HER2, that has been extensively investigated because of its potent oncogenic activity in several human carcinomas when overexpressed [149]. Trastuzumab, a monoclonal antibody against HER2, has become an important therapeutic option for patients with *HER2* amplification [149]. By FISH using probes for *HER2* and *TP53* genes, and centromere sequence for chromosome 17, that contains *HER2* and *TP53*, we analyzed both *HER2* and *TP53* in HCC CTCs isolated from 11 patients with five or more CTC counts [200]. The results (Fig. 12.3) showed that six patients (6/11) had *TP53* gene deletion, and 2 patients had chromosome 17 gain in apparent triploid background, both with biallelic deletion of *TP53*; two patients had *HER2* amplification, both having more than 50 of CTC counts. Among six patients with *TP53* gene deletion, one had *HER2* amplification. These genetic abnormalities were not detected in CTC samples from four patients. The results suggest that CTCs from various HCC patients or even from one HCC patient possess different genetic characteristics, and analysis of *HER2* in CTCs may have a potential in selecting patients for treatment with anti-HER2 mAb because of a frequency of amplification for *HER2* in CTCs.

In another study by Li et al. [95], triple-immunofluorescence staining was performed to examine the existence of EMT in CTCs from 46 patients with HCC, and their clinical relevance was analyzed. The expression of ZEB1, ZEB2, and snail could also be partially detected in CTCs, and E-cadherin and slug expression was absent in all CTCs. Of the 46 patients, 39 (84.8 %) had twist expression in CTCs, 37 (80.4 %) had vimentin expression, and 32 (69.6 %) had both coexpression. Either twist or vimentin expression in CTCs was significantly correlated with PVTT and TNM staging, vimentin expression in CTCs was significantly correlated with tumor size, and coexpression of both was highly correlated with PVTT, TNM staging, and tumor size. The results suggest that twist and vimentin expression in CTCs may serve as promising biomarkers for evaluating metastasis and prognosis in HCC patients.

Nel et al. [127] also used multi-immunofluorescence staining to detect CTCs with expression of mesenchymal markers such as vimentin and N-cadherin, and analyze their relationship with survival in HCC patients. The patients with

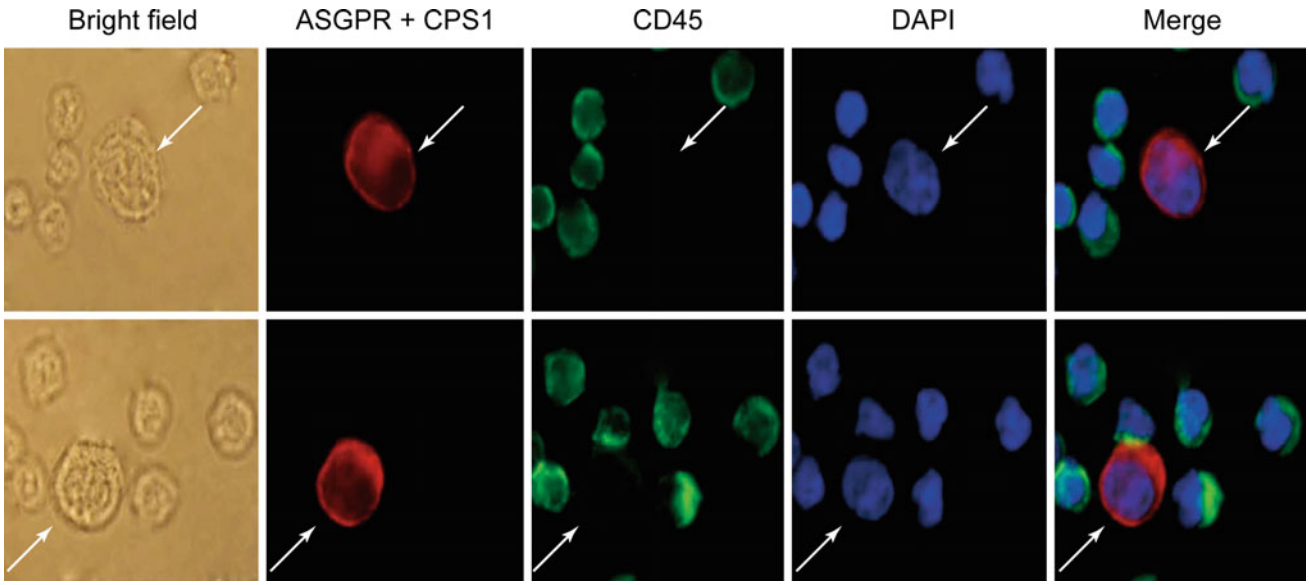

Fig. 12.3 Fluorescence in situ hybridization (FISH) on CTCs from patients with hepatocellular carcinoma. **a** The normal FISH signals in a CTC from patient 2 compared with the normal leukocyte from the same sample. *HER2* (*red*), *TP53* (*orange*), and the reference 17 centromere probe (*green*) are all present in 2 copies. **b** Monoallelic deletion of *TP53* seen as a single *orange* signal, with other probes showing normal copy number in this cell. **c** *HER2* amplification in a CTC from patient 8 with normal signals of both *TP53* and chromosome 17. **d** Biallelic deletion of *TP53* and chromosome 17 gain (*3 copies*) are present in patient 11, with 4 *orange* signals of *HER2* and a *HER2*/CEP 17 ratio of 1.3. Reproduced from Xu et al. [200]. Permission from Baishideng Publishing Group Inc

a vimentin(+)/CK(+) ratio of > 0.5 had a longer median TTP (1 vs. 15 months; $P = 0.03$) whereas the patients with a N-cadherin(+)/CK(+) ratio of < 0.1 had a shorter TTP (1 vs. 15 months; $P = 0.03$), suggesting a significant correlation between the shift from mesenchymal to epithelial cell profiles and shortened TTP in HCC patients.

Next-generation sequencing technologies can now sequence very small amounts of input DNA with high accuracy. Kelley et al. [83] performed targeted ion semiconductor sequencing on whole genome-amplified DNA from CTCs, PBMCs, and tumor specimens in HCC patients, and identified 86 variants overall from all of the CTC and tumor samples combined. Approximately 54 % variants were low-frequency, among which 93 % were from CTC samples ($P < 0.001$). Characteristic mutations in cancer (*TP53*, *PTEN*) were detected in CTC-derived DNA from two HCC cases. In one HCC case with matched CTCs, PBMCs, and tumor DNA, 8 SNPs were present and concordant in both PBMCs and tumor DNA; 5 of these (63 %) were identified in the CTC DNA.

Sorafenib is a multitargeted drug with multiple antitumor effects, and can improve the survival of patients with advanced HCC. However, not all patients respond equally well to sorafenib, and the response rate is relatively low [22, 33]. A number of studies have indicated that the inactivation of Ras/Raf/extracellular signal-regulated kinase (ERK) pathway and the activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway play a critical role in the resistance to sorafenib [33]. Recently, we investigated if phosphorylated ERK (pERK)/pAkt phenotyping of CTCs (Fig. 12.4) can be used as a biomarker to provide predictive information on response to sorafenib therapy [94]. After two weeks of sorafenib treatment, the counts of CTCs exhibited a shaper decline in patients with pERK(+)/pAkt(−) CTCs ($P < 0.01$). Disease control rates were significantly different between patients with pERK(+)/pAkt(−) CTCs (73.3 %) and those without (29.5 %) ($P < 0.05$). Univariate and multivariate analysis showed pERK(+)/pAkt(−) CTCs as an independent predictive factor of progression-free survival (PFS) (hazard ratio = 9.389; $P < 0.01$). PFS correlated with the proportion of pERK(+)/pAkt(−) CTCs (r = 0.968, $P < 0.01$), and was higher in patients with ≥ 40 % pERK(+)/pAkt(−) CTCs compared to those with < 40 % (8.4 vs. 1.3 months; $P < 0.05$) (Fig. 12.5). In a validation set of 20 HCC patients, CTCs from patients with ≥40 % pERK(+)/pAkt(−) CTCs had significantly higher inhibition rates of spheroid formation compared to those with < 40 % (61.2 vs. 19.8 %; $P < 0.01$) (Fig. 12.6). These finding suggested that in HCC patients treated with sorafenib, pERK(+)/pAkt(−) CTCs are most sensitive to sorafenib and an independent predictive factor of PFS.

12.8 Targeting of HCC CTCs for Prevention of Postoperative Recurrence and Metastasis

The current curative therapies for HCC are limited to the surgical removal of tumors or liver transplantation. However, postoperative recurrence and metastasis are common complications [27–30, 50, 106, 112, 113, 123, 146]. Many studies have associated the presence of either preoperative or postoperative CTCs with an increased risk for HCC recurrence, and CTCs are increasingly recognized as the main source [178, 209, 211, 212]. Clinicians must therefore be aware of this when treating patients with HCC and should collaborate with other researchers to develop and employ novel therapeutic techniques that target HCC CTCs in different stages throughout the course of treatment to prevent and reduce the postoperative recurrence and metastasis. To this end, a personalized, comprehensive, and multidisciplinary strategy should be considered [209, 212].

12.8.1 CTCs as an Indication for Curative Surgery in HCC

Aside from the contraindication of extrahepatic metastasis, no uniform screening criterion has been established for surgical resection. Based on tumor size, nodule number, and degree of vascular invasion, HCC patients are selected for liver transplantation. Because these criteria cannot accurately define the rational distribution of the donor liver and predict the prognosis of the patient, there is controversy over which of these are most appropriate and feasible [113]. It is therefore necessary to incorporate more objective and reliable laboratory indexes beyond the clinicopathologic criteria for HCC. If a high preoperative level of CTCs can predict the true benefit of curative treatment and a prognosis, it may prove to be a reasonable candidate index for surgical indications. To this end, intensive cooperation among worldwide leaders in this field should be encouraged to verify whether CTCs can be used as an objective indicator or contraindication for curative hepatectomy, as well as to reach a consensus on assays and result reporting.

12.8.2 Reduction of Basal CTC Levels with Preoperative Neoadjuvant Therapy

Early detection of metastatic spread provides an opportunity for perioperative administration of therapeutic agents. However, due to the underlying liver disease present in

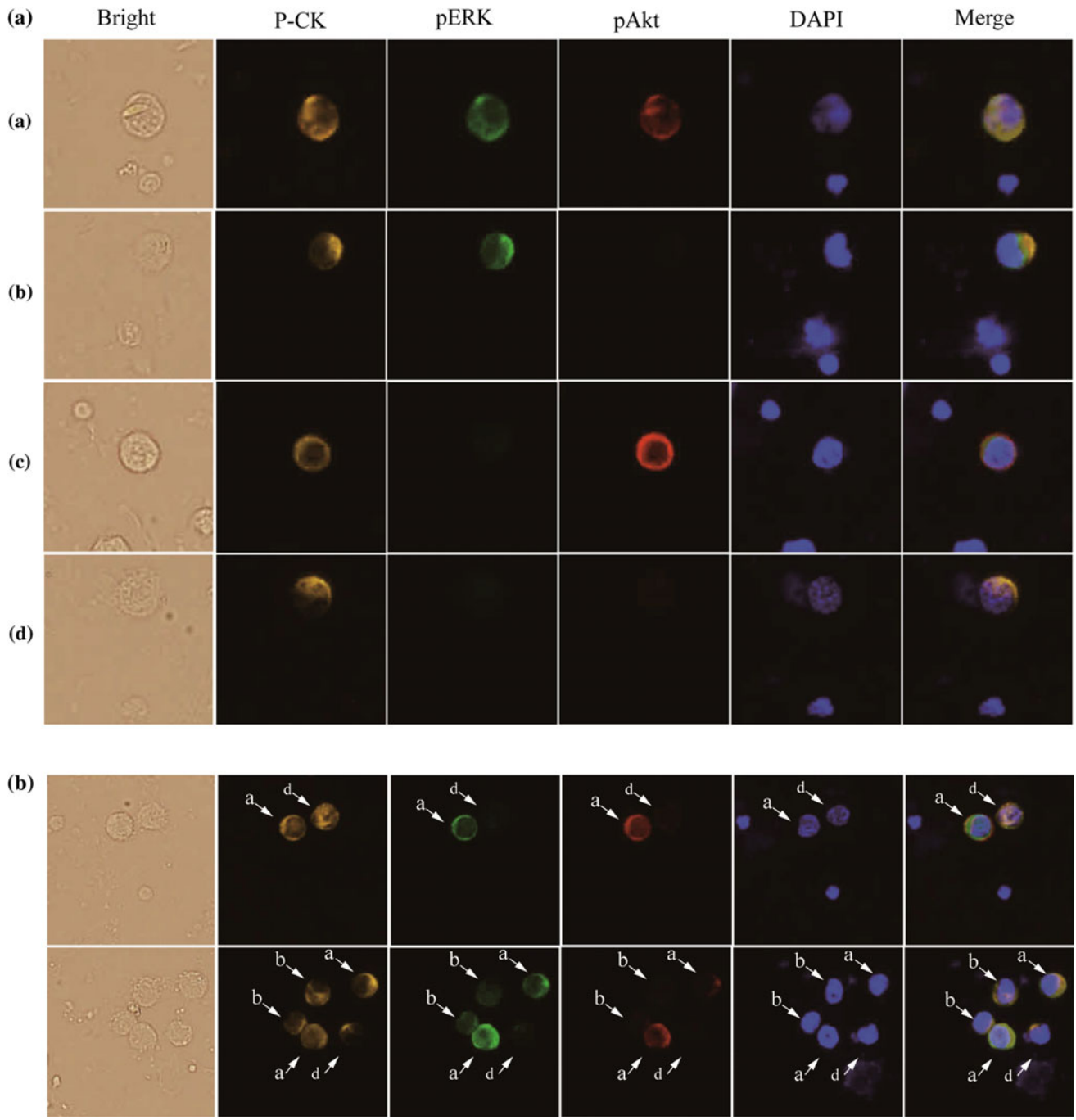

Fig. 12.4 Detection of phosphorylated extracellular signal-regulated kinase (pERK) and protein kinase B (pAkt) in circulating tumor cells (CTCs) of hepatocellular carcinoma (HCC). **A** CTCs stained for pan-cytokeratin (P-CK) (*yellow*), pERK (*green*), pAkt (red), and contained with 4′,6-diamidino-2-phenylindole (DAPI) (*blue*) (*magnification* ×400). **B** Coexistence of CTCs with various patterns of pERK/pAkt in the same field of view detected by multicolor immunofluorescence staining (*magnification* ×200). **a** pERK(+)/pAkt(+); **b** pERK(+)/pAkt(−); **c** pERK(−)/pAkt(+); **d** pERK(−)/pAkt(−). Reproduced from Li et al. [94]

almost all patients and the insensitivity of HCC cells to chemotherapy drugs, adjuvant therapy in HCC represents a great challenge, and thereby currently is no standard of care for preoperative neoadjuvant therapy [29, 50, 146]. Sorafenib, as a multikinase inhibitor for treatment of advanced HCC, has provided promising results. Some of clinical

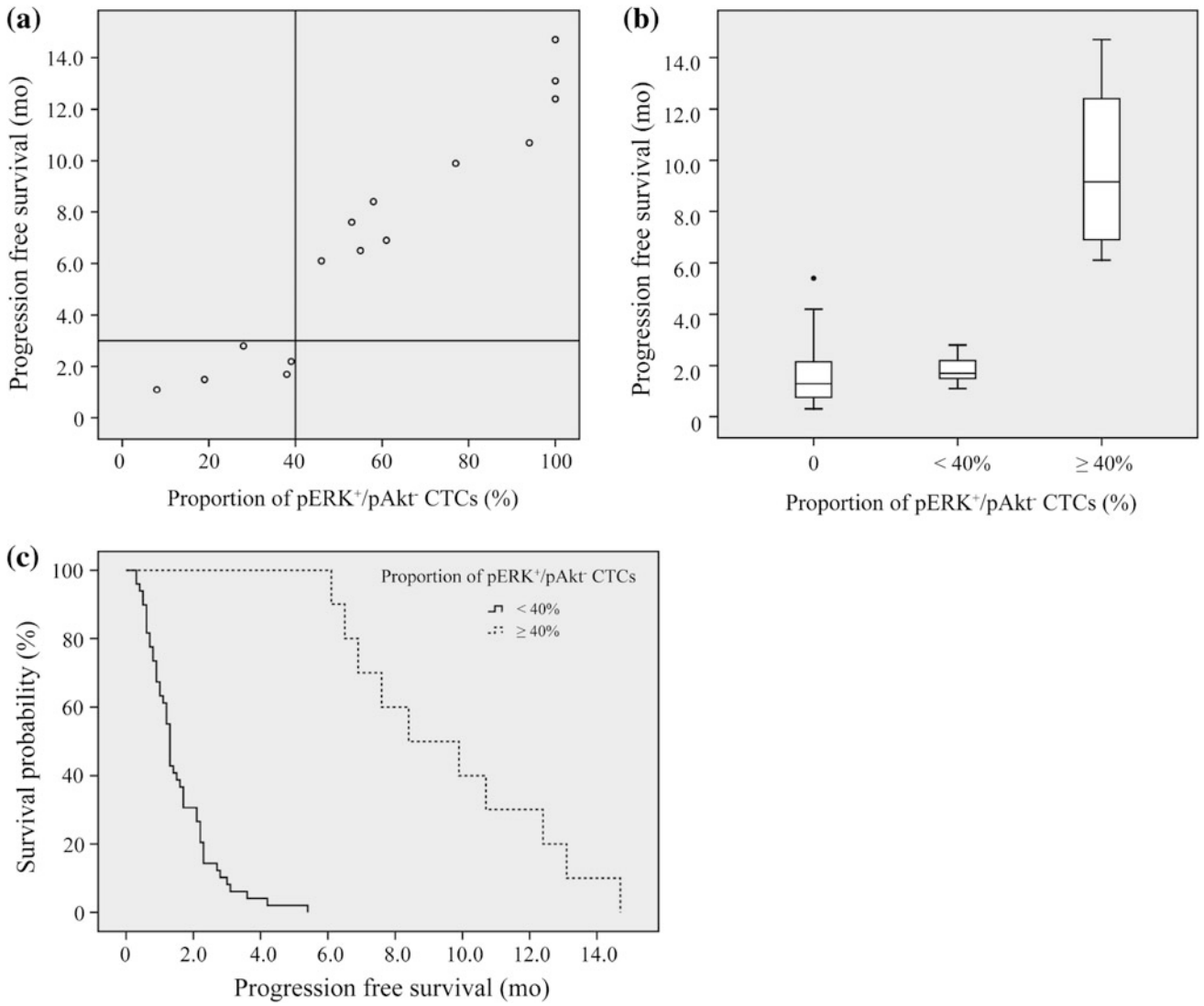

Fig. 12.5 Phosphorylated extracellular signal-regulated kinase (pERK)(+)/protein kinase B (pAkt)(−)/total circulating tumor cells (CTCs) [pERK(+)/pAkt(−)/CTCs] as a potential predictive factor of patients with (HCC) receiving sorafenib therapy. **a** Progression-free survival after sorafenib treatment in patients ($n = 15$) according to pERK(+)/pAkt(−)/CTCs. **b** Progression-free survival in patients with ≥ 40 % ($n = 10$) or < 40 % ($n = 5$) of CTCs identified as pERK(+)/pAkt(−), and those without pERK(+)/pAkt(−) CTCs ($n = 44$). **c** Survival curves of patients with ≥ 40 % ($n = 10$) or < 40 % ($n = 49$) of CTCs identified as pERK(+)/pAkt(−). Reproduced from Li et al. [94]

studies indicate that as a preoperative neoadjuvant therapy, sorafenib was shown to downstage HCC, likely providing opportunities for curative treatment [17, 75, 196]. For HCC patients waiting for liver transplantation, sorafenib is cost-effective [184]. We recently investigated the effect of sorafenib on CTC levels, and found that all patients showed a decrease in CTC counts after two weeks of sorafenib monotherapy [94]. Since the risk of postoperative recurrence increases with the level of preoperative CTCs, preoperative neoadjuvant therapy for elimination of CTCs could theoretically reduce the risk. Therefore, clinical studies on sorafenib and other neoadjuvant therapies preoperatively targeting CTCs are needed to evaluate their effects on the risk of postoperative recurrence and metastasis.

12.8.3 Proper Surgical Techniques for Minimizing the Intraoperative Release of CTCs

An important principle of surgical oncology is that prevention of postoperative recurrence and metastasis requires eliminating iatrogenic tumor cell seeding during the surgical

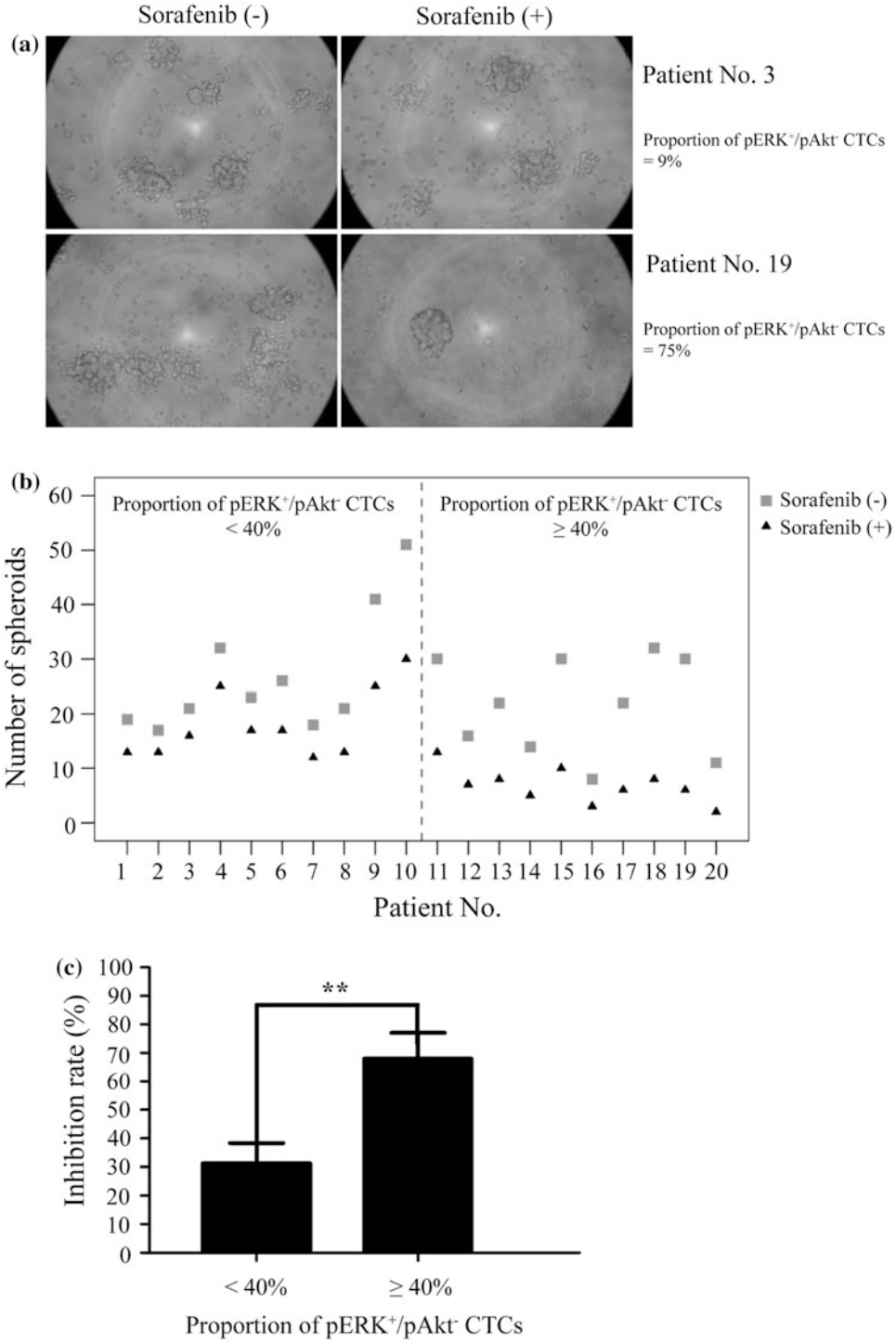

Fig. 12.6 Sensitivity of circulating tumor cells (CTCs) to sorafenib. CTCs isolated from patients with hepatocellular carcinoma (HCC) ($n = 20$) were tested by spheroid formation assay. **a** CTCs from two patients formed spheroids at day 7 in culture with or without sorafenib. **b** Formation of spheroids of CTCs treated with or without sorafenib. **c** Spheroid formation inhibition rates from patients with $\geq$ 40 % or < 40 % of CTCs identified as pERK(+)/pAkt(−). Reproduced from Li et al. [94]

operation, when cancer cells are more easily found in surrounding blood circulations, especially in the venous blood from the tumor. As a result, a preoperative absence of CTCs can be converted into a postoperative presence. Improper surgical manipulation such as squeezing or traction of the liver tumor may promote the dissemination of cancer cell into the blood circulation. For example, due to moving the liver, the conventional posterior approach to hepatic lobectomy likely results in squeezing of the liver and the release of cancer cell. By contrast, the anterior approach to a right hepatic lobectomy, without lifting and squeezing of the liver likely reduces or avoids the release of CTCs. This free-tumor technique was first devised by Lai et al. [89] and later modified by Belghiti et al. [20], thereby developing the liver

hanging maneuver for hemihepatectomy. Retrospective or prospective studies have demonstrated that this surgical modality is a practical and can effectively reduce postoperative recurrence [77, 198]. Likewise, during liver transplantation, the "no-touch" technique should also be advocated for removal of the diseased liver. In a word, surgical oncologists should avoid any improper surgical procedures and minimize hematogenous metastasis of cancer cells during surgery.

12.8.4 Postoperative Adjuvant Therapy for Elimination of Residual CTCs

The results of studies on CTC dynamics in orthotopic HCC models revealed that the numbers of CTCs and early metastases decrease significantly after resection [52, 155, 156], and clinical studies also indicated similar results [16, 51, 58, 95, 158, 168, 200]. However, radical hepatectomy or liver transplantation cannot eliminate all preoperatively existing CTCs. Obviously, surgical intervention should not be considered the treatment endpoint, and postsurgical targeting of CTCs should thus be addressed. A study in an orthotopic mouse model showed that postoperative use of sorafenib suppressed the development of intrahepatic recurrence and abdominal metastasis, prolonging postoperative survival [53]. Results from a pilot study of HCC patients also indicated that adjuvant therapy with sorafenib prevented early postoperative recurrence [192]. Another single-center experience showed that adjuvant sorafenib did not decrease tumor recurrence, but significantly reduced mortality and prolonged overall survival of HCC patients after curative resection [209, 212]. However, a randomized phase 3, double-blind, placebo-controlled study of sorafenib as adjuvant treatment after potentially curative therapy for HCC showed no significant treatment effect with sorafenib, with regard to recurrence-free survival (RFS), time to recurrence, or overall survival [28, 30]. For liver transplantation, treatment of a rodent HCC model with sorafenib effectively inhibited recurrence and metastasis, without a negative influence on the immune balance [202]. Furthermore, a clinical study also suggested that sorafenib delayed or reduced posttransplant recurrence and prolonged patient survival [70].

In spite of these results, further clinical studies should be conducted on the therapeutic targeting of CTCs as a postoperative adjuvant therapy. It should be noted that some of CTCs may remain in a nondividing state for a long period of time, or may never divide, and are thus insensitive to chemotherapy or molecular targeted therapy. Therefore, targeting of CTCs by using antibody-based immunotherapy probably represents a promising avenue for future research.

12.8.5 Dynamic Postoperative Monitoring of CTCs for Guiding the Therapeutic Decisions

Since CTCs could be released during surgical operation, it is also important to accurately assess their levels after removal of the tumor load. A close follow-up of patients for early signs of recurrence and metastasis allows for early treatment intervention. Theoretically, CTCs should be therapeutically targeted in advance. For patients treated with chemotherapy and/or radiotherapy, CTCs could be regularly monitored to assess therapeutic outcome. In addition, CTCs can be used to identify the targets of sorafenib before making a decision about administration, as well as to detect resistance to sorafenib during administration. In this respect, we recently present a unique platform to provide quantitative information concerning sorafenib-related targets in CTCs, define the molecular subtypes of HCC to identify patients particularly susceptible to sorafenib, and predict drug response and efficacy [94]. As a result, patients most likely to benefit could be selected, ultimately increasing the success of sorafenib treatment, while preventing unnecessary treatments, serious side effects, and high costs.

12.9 Future Perspectives

12.9.1 Detection Techniques of CTCs

In order to translate the detection of HCC CTCs into the clinic, the refinement of existing technologies or the innovation of multiplexing approaches will be required to create high-throughput, reliable, and cheap platforms. However, we will continue to face major technical challenges. The development of novel strategies for CTC capture is a particularly important aspect. If a specific marker is used for that, it would ideally be expressed on the surface of every cancer cell. In fact, such a biomarker is unavailable so far, and as a result, current marker-based CTC capture approaches may lead to lower yield and purity. So it is imperative to find novel markers, biological or physical, that can specifically detect all subpopulations of HCC CTCs.

It should be noted that there has been much confusion about how to identify a CTC. CTCs are considered highly heterogeneous and also probably undergoing apoptosis. The marked heterogeneity exists even within a distinct or same histological tumor type [13, 159], that makes it difficult to precisely define CTCs. There is currently no single parameter sufficient to define the "true" CTCs, such as size, cytomorphology, and pathology stains; the used parameters may show overlap with normal controls or leukocytes. In addition, due to the lack of a detectable characteristic such as

EpCAM, CKs, and AFP, some of CTCs may escape existing technologies [191]. Perhaps importantly, sensitivity and specificity may not be just technical but biological as well. Not all detected cells are bad, and not all bad cells are detected [195]. Therefore, different definitions for CTCs may lead to different results of CTC enumeration with varying clinical relevance. Clearly, a "single" or "standard" definition for CTCs is clinically important.

12.9.2 Clinical Relevance of CTCs or CCSCs

Over the past decade, CTCs has gained considerable attention. Some studies [16, 51, 58, 95, 158, 168, 200] have been conducted on CTCs or so-called "CCSCs" in HCC patients, and indicate that CTCs or CCSCs correlate with poor prognosis in patients with HCC, and may contribute to postoperative recurrence and metastasis, thereby serving as an important therapeutic target. These results look encouraging. However, the analyses were limited by small sample sizes, short follow-up time, and data from a single study center, as discussed by the authors of these studies. Therefore, a clinical trial including a larger number of patients from multicenter as well as prospective long-term follow-up data is needed to demonstrate and validate the clinical utility of these "CCSCs" in HCC, especially EpCAM(+) CTCs.

Another limitation is probably that these studies used different CCSC definitions (CD90(+)CD44(+) cells and EpCAM(+) cells [51, 58, 158, 168, 205, 206], and detected different numbers of CCSCs. Even using the same method of the CellSearch system, Sun et al. [168] identified ≥ 1 CTC in 82/123 (66.67 %) patients, with a range of 1–34 CTCs, whereas Morris et al. [122] identified ≥ 1 CTC in 14/50 (28 %) patients, with a range of 1–8 CTCs. Furthermore, definitive liver CSC markers remain controversial. CSCs enriched by different methods exhibit phenotypic and genetic heterogeneity including expression of different stemness markers but share similar CSC properties [107, 152, 201]. Finally, the overlap among most markers is very low, and liver CSCs do not exclusively express a single marker [23, 117]. Thus, in order to develop technologies capable of reliably isolating and characterizing liver CCSCs, it is necessary to identify additional specific markers for putative liver CSCs, or to further validate CD90 or CD44 or EpCAM as a definitive liver CSC marker.

It remains to be seen whether there is a "one-fits-all" marker for CSCs in HCC, or how much overlap there is between various markers that have been used for CCSCs in HCC [63]. Therefore, considerable attention should then be paid to identify mesenchymal and stem-like cells, and MICs among HCC CTCs, including optimization of methods for CTC detection with EMT- or stemness-related markers, colony formation assay, xenotransplantation of the phenotypically selected and in vitro expanded CTCs into experimental models, isolation of enough CSCs clonally derived from primary tumor without in vitro propagation for differentiation and lineage tracking experiments, and identification of deregulated signal transduction pathways [63]. In a word, in order to develop technologies capable of reliably isolating and characterizing liver CCSCs, it is necessary to identify additional specific markers for putative liver CSCs, or to further validate CD90 or CD44 or EpCAM as a definitive liver CSC marker. The knowledge from these studies will open up new avenues for the development of new targeted therapies aimed at those highly aggressive cells.

12.9.3 Molecular Characterization of CTCs

HCC is often a heterogeneous and multifocal disease. Evidence is accumulating that CTCs are also highly heterogeneous, and different subpopulations of CTCs might exhibit different properties [147]. "Just", enumeration of CTCs cannot detect the ability of a cancer cell to invade, proliferate, and cause a metastasis. Therefore, it would be of great value to functionally characterize them beyond enumeration [195]. Molecular characterization of CTCs will address the following issues: Whether genetic alterations in CTCs more truly reflect the situation of particular metastatic tumors? Whether early disseminated CTCs acquire new genomic aberrations allowing for their growth? Which characteristics of CTCs may contribute to metastatic properties, or affect clinical outcome? What changes in the genotype of CTCs have occurred over the course of therapy? The insights from these studies will improve our knowledge about the biology and mechanisms of recurrence and metastases in HCC patients, and probably promote the identification of one or more new and ideal markers that can hopefully be used to design more effective anticancer drugs, select effective targeted therapies and unravel resistance mechanisms. So far, only limited markers have been analyzed on isolated CTCs by several methods. Certainly, detail characterization of CTCs will depend on more sophisticated analytical approaches. Recent advances in biotechnology have allowed the comprehensive analysis of the whole-gene or protein expression profile from hypocellular samples. Particularly, developments in next-generation sequencing and whole genome amplification have enabled genomic analyses of single cells. As for single-cell sequencing, sequencing mutations or genomic profiling of copy number in CTCs will be one of the major clinical applications, and the results may identify thousands of potentially aberrant cancer genes from

a fuller picture of a single-cell genome, such as deletions of tumor suppressors and genomic amplifications of oncogenes, thereby providing clinicians with the information necessary to direct precise therapy or monitor disease over time [125].

12.10 Conclusions

HCC is one of the most aggressive cancers. Surgery for HCC has been performed for more than 100 years, including a 40-year history of liver transplantation [91]. However, the outcome of surgical therapy alone has not improved. Similar to CTCs from other cancer types, HCC CTCs are increasingly recognized as the main source for postoperative recurrence and metastasis. In the case of tumor removal or liver transplantation, CTCs may return to the liver remnant or the newly implanted healthy liver and initiate intrahepatic recurrence.

CTCs hold promise as a research tool to improve our knowledge of multiple metastatic steps and identify novel therapeutic targets, and as a minimally invasive, real-time "liquid biopsy" for the identification of biomarkers to aid in detecting early cancer, predicting prognosis, monitoring therapy response, and selecting drugs for patients. During the last decades, isolation and detection of CTCs has attracted increasing interest and has led to a wide range of laboratory and commercial technologies. However, the translation of CTCs to a routine clinical test is hindered by the lack of validation and qualification of these multiple different technologies. Although the CellSearch system has been approved for clinical practice as a useful prognostic biomarker, EpCAM-based methods of CTC isolation may cause some bias against some tumor cells. Therefore, efforts now need to focus on development of more sensitive technologies to accommodate CTC heterogeneity. Recently, the field has expanded beyond enumeration and a lot of work is focused on further detection of CTC subpopulations including CTM, mesenchymal and stem-like CTCs, and MICs in order to investigate their functional biology and metastatic potential. Thus, more flexible methods for functional characterization of CTCs, including single CTC analysis and their ex vivo expansion and xenotransplantation are also needed.

Since only a small percentage of HCC cases are positive for EpCAM, EpCAM-dependent strategies are not unsuitable to capture CTCs in HCC patients. Probably for this reason, not a few studies on CTCs have so far been conducted in HCC patients, and knowledge about clinical relevance of HCC CTCs is lagging behind other major types of cancer, such as breast, prostate, colon, and lung cancer. Fortunately, some interesting and encouraging achievements have been made in this field, and the situation has started to change for HCC. Clinicians must pay attention to CTCs when treating HCC patients. In this chapter, we proposed some HCC CTC-based strategies for the management of HCC according to the recent literature. In order to prevent and reduce the postoperative recurrence and metastasis, intensive collaboration with other researchers is currently needed to develop and employ novel and effective therapeutic techniques that target CTCs. We believe that the application of appropriate diagnostic and therapeutic approaches targeting HCC CTCs in different stages over the entire clinical course or at least throughout the course of treatment may represent a major breakthrough in preventing the postoperative recurrence and improving the therapeutic outcome of HCC.

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Role of the Immune System in Hepatocellular Carcinoma: Implications for Existing and Novel Therapies

13

Tobias Flecken and Robert Thimme

Contents

13.1 The Immune System and Development of HCC

Chronic hepatitis represents a common pathway shared by most HCCs [1]. This chronic inflammation of the liver comprises many cells and molecules of the immune system that actively contribute to the development of HCC [2]. This could be clearly demonstrated in murine models. For example, if immune cells from HBV-vaccinated mice were transferred into mice expressing the surface antigen of HBV (HB_SAg) in the liver, this was sufficient to induce HCC in the absence of HBV infection or replication [3]. Even more strikingly, hepatic overexpression of the cytokine lymphotoxin-β (LT-β) alone was sufficient to promote the development of HCC in mice [4]. Recent work could also demonstrate that the occurrence of HCC in a murine model of nonalcoholic steatohepatitis (NASH) is dependent on activation of and cytokine secretion by $CD8^+$ T cells and natural killer T (NKT) cells [5].

The situation is clearly more complex in affected patients. Here, tumorigenesis occurs over a much longer period and is likely multifactorial. Accordingly, several pathways have been demonstrated to affect the development of HCC. Many inflammatory cell types of the immune system, such as macrophages/Kupffer cells, monocytes, or granulocytes can produce reactive oxygen and/or nitrogen species (ROS/RNS). ROS/RNS can have potent antimicrobial effects but they can also directly damage hepatocytes and their genetic material, inducing mutagenesis [6]. Indeed, intrahepatic oxidative stress after clearance of HCV by interferon (IFN)-based therapy has been shown to be an independent prognostic factor for the development of HCC in these patients [7]. This increased oxidative stress not only damages cells but also affects molecules in their environment. Molecules modified by ROS/RNS may trigger pattern-recognition receptors (PRR) of the innate immune system, which usually have an important role in sensing the presence of pathogens and providing appropriate "danger" signals to induce an immune response. Continued activation of PRR

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by ROS/RNS promotes inflammation and thus leads to a further increased generation of ROS/RNS, resulting in a vicious cycle of chronic inflammation [8].

PRR such as Toll-like receptors (TLR) have been described to directly promote hepatocarcinogenesis. Recent work performed in a murine model of HCC could demonstrate that lipopolysaccharide (LPS) derived from the intestinal microbiota acts via TLR4 on hepatocytes [9]. Gut sterilization by high-dose antibiotic treatment resulted in a reduced number and size of HCC lesions, mediated by a loss of antiapoptotic effects of TLR4 signaling. TLR4 signaling may also promote generation of cancer stem cells as well as epithelial–mesenchymal transition (EMT) and thus metastasis of HCC [10, 11]. However, the picture of TLR in HCC appears to be more complex. TLR represent a family of receptors where individual members may have distinct roles. One study described TLR3 expression to correlate with increased apoptosis and reduced proliferation, vascularization, and invasiveness of human HCC [12]. Another study described an increased development of HCC in TLR2$^{-/-}$ mice [13]. While TLR as a major component of innate immunity have a complex role in HCC, further research is clearly required.

The antiapoptotic properties of TLR signaling mainly depend on activation of the transcription factor nuclear factor-κB (NF-κB) in hepatocytes [14, 15]. While activation of NF-κB in acute inflammation protects hepatocytes from ROS and inflammation-induced damage, its prolonged activation during chronic inflammation results in hepatocarcinogenesis, e.g., by impairing clearance of damaged hepatocytes by apoptosis [4, 16]. NF-κB also has an indirect role in driving the development of HCC. Death of hepatocytes can result in the release of interleukin-1α (IL-1α), which is a potent activator of NF-κB in surrounding immune cells, e.g., Kupffer cells or tumor-associated macrophages (TAM) [16, 17]. In these cells, NF-κB activation drives the production of other proinflammatory cytokines, most importantly IL-6. In patients with chronic HBV and HCV infection, serum IL-6 levels were significantly correlated with the risk of HCC [18, 19]. Notably, males typically exhibit higher serum levels of IL-6 at baseline as well as in HCC compared to females, providing a mechanistic explanation for the higher frequency of HCC in males [20]. IL-6 can directly promote growth of HCC by activation of another important pathway in hepatocytes, signal transducer, and activator of transcription-3 (STAT3) [16]. Early HCC precursors were shown to highly depend on IL-6-mediated STAT3 activation [21]. In addition, the IL-6/STAT3 axis appears to particularly promote cancer stem cells, also in later tumor stages [22, 23]. Other cytokines such as transforming growth factor-β (TGF-β) are also important players. TGF-β has a dual role in HCC, promoting carcinogenesis in early stages but suppressing proliferation in established HCC [2]. TGF-β also exerts indirect effects by induction of a special population of TAM that express T-cell immunoglobulin-domain and mucin-domain containing molecule-3 (Tim-3). Tim-3-expressing TAM in turn promote HCC growth by secretion of IL-6 [24]. TAM were also shown to enhance EMT and invasiveness of human HCC by secretion of CCL22 and IL-8 [25, 26]. They can also increase cancer stem cell proliferation by TGF-β [27]. Hence, intratumoral TAM have been shown to correlate with a poor prognosis [26].

Together with IL-6, TGF-β also promotes production of IL-17 by cluster of differentiation 4 (CD4)$^+$ T helper cells, the central regulators of adaptive immunity, and CD8$^+$ cytotoxic T cells [28]. These T_H17 and T_C17 cells are strongly proinflammatory T-cell subsets that are important for clearing extracellular bacteria but have also been suggested to contribute to control of fungi. Human monocytes that were incubated with supernatants from HCC cell lines were highly efficient in inducing T_H17 and T_C17 cells [29, 30]. Despite their strong proinflammatory role, tumor-infiltrating IL-17 producing cells are associated with poor survival of HCC patients [31]. Multiple factors contribute to this pro-tumorigenic role of T_H17 and T_C17 cells. IL-17 producing cells function in the early stages of adaptive immune responses to recruit granulocytes to the site of inflammation, a process that is recapitulated in HCC in a chronic fashion. Here, the occurrence of peritumoral neutrophilic granulocytes is associated with the occurrence of IL-17 producing cells [32]. These peritumoral neutrophils function by remodeling the surrounding tissue in a manner that favors motility and thus invasion as well as spread of HCC. They may also induce angiogenesis, as suggested by the effects of neutrophils in in vitro co-culture systems [32, 33]. This pathway is most likely also relevant in vivo, since the density of IL-17 producing cells correlates with microvessel density in human HCC [31]. In addition to these indirect effects, T_H17 cells can also produce other cytokines such as IL-22. Similar to IL-6, IL-22 can promote the activation of STAT3 in hepatoma cells and thus directly promote tumor growth [34]. Interestingly, a recent study could describe the occurrence of a specialized CD4$^+$ T-cell subset producing IL-22 but not IL-17, termed T_H22 cells, specifically in the tumor of HCC patients [35]. The frequency of these cells was increased in later stage tumors and in intrahepatic metastases, in line with their likely role in promoting tumor growth. Even though a mechanistic role of IL-22 was not investigated in this study, it appears likely that these cells may also promote HCC growth by IL-22-mediated activation of STAT3.

Altogether, the described work has demonstrated that several components of the immune system have a direct role in promoting the development and progression of HCC. Even though several pathways have been elucidated, research is still ongoing and needed to deepen our

understanding of the inflammation-induced development of HCC and the role of different elements of the immune system in this process. However, the immune system also has the potential to limit the growth of HCC. This other side of the immune system's dual role in HCC will be discussed in the following section.

13.2 Success and Failure of Immune Control in HCC

Several lines of evidence support an antitumor role of the immune system in HCC. A lymphocytic infiltrate in human HCC lesions correlates with tumor cell apoptosis and reduced recurrence of HCC after hepatic resection and liver transplantation, respectively [36–38]. Especially, the occurrence of tumor-infiltrating effector $CD8^+$ T cells that produce the cytokine IL-33 as well as cytotoxic molecules was shown to correlate with improved patient survival [39]. Additionally, work in murine models of HCC has shown that the immune system can remove malignantly transformed cells in the liver before they develop into HCC lesions, a process often termed immunosurveillance [40, 41]. Immunosurveillance has been shown to be mediated either by the action of T cells or by macrophages that are activated by $CD4^+$ T helper cells, depending on the model used. Macrophages were found to be particularly important in a model relying on hydrodynamic tail-vein injection of plasmids encoding a mutant form of the proto-oncogene n-Ras to promote hepatocyte transformation [40]. However, this method only generates very weak $CD8^+$ T-cell responses if plasmids encoding viral proteins are used and may hence underestimate the contribution of $CD8^+$ T cells to immunosurveillance in HCC [42]. This is important to note since $CD8^+$ T cells are the main effector cells of cell-mediated adaptive immunity. They recognize peptides derived from intracellular proteins, which are presented on the surface of all somatic cells on major histocompatibility complex (MHC) proteins and can perform multiple effector functions including secretion of cytokines, such as IFN-γ, as well as cytotoxic lysis of target cells, e.g., by release of granzyme B and perforin [43]. To avoid destruction of healthy tissues, the activation of $CD8^+$ T cells is tightly regulated and requires professional antigen-presenting cells (APCs), such as dendritic cells (DCs). These APCs have to be activated by strong inflammatory signals and/or $CD4^+$ T helper cells. Additionally, $CD8^+$ T cells recognizing "self" peptides presented on MHC molecules are deleted during their development to prevent autoimmunity. $CD8^+$ T cells are nevertheless capable of recognizing malignantly transformed cells because these express special tumor-associated antigens (TAA) [44]. TAA comprise proteins that are either mutated or grossly overexpressed in tumors compared to healthy, adult tissues and hence not or only partially subject to $CD8^+$ T-cell tolerance. Various TAA could be identified in HCC as well as other types of cancer, including α-fetoprotein (AFP), glypican-3, human telomerase-reverse transcriptase (hTERT), or New York-esophageal squamous cell carcinoma-1 (NY-ESO-1) [44]. Subsequent comparative studies indicated that the frequency of $CD8^+$ T-cell responses to individual TAA is quite variable, depending on the cohort analyzed and the MHC molecules presenting the TAA-derived epitopes [45–47]. Importantly, co-expression of multiple TAA in HCC lesions has been found to correlate with improved overall survival of HCC patients [48]. In addition, the occurrence of TAA-specific $CD8^+$ T-cell responses is also associated with improved recurrence-free survival in HCC patients [47, 49]. In line with this apparent antitumor effect in HCC patients, expression of perforin ex vivo and cytotoxic capacity after in vitro expansion could be demonstrated for $CD8^+$ T cells specific for the TAA melanoma-associated gene-A3 (MAGE-A3) in some HCC patients [50, 51].

Despite their potent effector functions, $CD8^+$ T cells fail to clear HCC in most patients. Accordingly, several studies have reported a dysfunction of TAA-specific $CD8^+$ T cells in patients with HCC, indicated by a reduced production of cytokines such as tumor necrosis factor (TNF) or IFN-γ [47, 52]. This was reflected by a decreased expression of the stimulatory molecules CD3ζ and CD28 and a concomitantly increased activity of proapoptotic caspase-3 in peripheral T cells from HCC patients [53]. In mice bearing virus-induced HCC, failure of HCC-specific $CD8^+$ T-cell immunity has been linked to expression of the inhibitory receptor programmed death-1 (PD-1) on $CD8^+$ T cells [54]. PD-1 can inhibit $CD8^+$ T-cell functionality after engagement by one of its ligands, most prominently programmed death-ligand 1 (PD-L1) [55]. In patients, the expression of PD-1 on $CD8^+$ T cells as well as the expression of PD-L1 on tumor cells is linked to an increased risk of HCC progression [56, 57]. Next to PD-1/PD-L1, other inhibitory receptors have also been shown to affect $CD8^+$ T cells in HCC patients. Tim-3 expression on tumor-infiltrating T cells is increased and correlates with reduced patient survival [58]. Furthermore, the inhibitory receptor lymphocyte activation-induced gene-3 (Lag-3) has been shown to suppress the function of tumor-infiltrating, HBV-specific $CD8^+$ T cells in chronically HBV-infected patients with HCC [59].

In addition to inhibitory receptors, $CD8^+$ T-cell extrinsic mechanisms also regulate the function of $CD8^+$ T cells, as briefly mentioned above. This is also reflected in HCC, where multiple additional pathways may also contribute to $CD8^+$ T-cell dysfunction. One important aspect may be a dysregulation of DCs in HCC patients. Indeed, several studies report a reduced expression of IL-12 by DCs from HCC patients [60, 61]. IL-12 is particularly important for the

initial activation (priming) of $CD8^+$ T cells [43]. While the production of IL-12 in DCs is reduced, the expression of several inhibitory molecules, such as IL-10 or indoleamine-2,3-dioxygenase (IDO), is conversely increased in patients with HCC [60, 62]. The stimulatory capacity of these DCs from HCC patients was accordingly reduced. One of the HCC-derived factors contributing to the observed changes in DCs may be AFP, which is secreted in large amounts by many HCCs [63]. This effect has been shown to depend on a tumor-specifically altered glycosylation pattern of AFP, explaining differences to earlier studies that did not observe any effects of normally glycosylated AFP on DCs [64].

For their proper activation, DCs require stimulation by $CD4^+$ T helper cells, as mentioned above. In HCC patients, tumor-specific $CD4^+$ T-cell responses appear to be of very low frequency [65, 66]. Alternatively, tumor-specific $CD4^+$ T cells may be functionally diverted and thus provide insufficient support to DCs and $CD8^+$ T cells. Indeed, a group of $CD4^+CD25^+$Forkhead box P3 $(FoxP3)^+$ regulatory T cells (T_{reg}) has been described. T_{reg} may exert suppressive effects by different mechanisms such as secretion of inhibitory molecules, cytotoxic T lymphocyte angiten-4 (CTLA-4)-mediated reduction of the costimulatory capacity of APCs, direct cytotoxicity against effector T cells, or metabolic inhibition of these cells [67]. An increase in T_{reg} frequencies has been observed in the tumor and liver of HCC patients, in some studies also in the periphery [47, 68, 69]. T_{reg} infiltration has also been linked to suppression of $CD8^+$ T cells and correlated to reduced overall and progression-free survival of HCC patients [70, 71]. T_{reg} may be induced indirectly in HCC by myeloid-derived suppressor cells (MDSC), a population of immature cells derived from the bone marrow that is expanded in patients with HCC as well as other cancers and possesses immunosuppressive capabilities [72]. Depletion of T_{reg} can enhance proliferation of tumor-specific $CD8^+$ T cells from HCC patients in vitro [47, 73]. Co-depletion of MDSC, T_{reg}, and PD-1^+CD4^+ T cells even resulted in a restoration of granzyme B production by $CD8^+$ T cells from HCC patients [74]. Thus, T_{reg} and other factors may be a central part of the immunosuppressive environment inhibiting tumor-specific $CD8^+$ T-cell responses.

Next to $CD8^+$ T cells, other cells of the immune system also have the ability to attack HCC, such as natural killer (NK) cells. NK cells constitute a major population in the liver and also have direct antihepatoma effects in vitro [75]. NK-cell activation is controlled by a balance of signals derived from a variety of activating and inhibitory receptors on the cell surface. Due to changes in the balance of signals from both types of receptor, NK cells can for example lyse cells that have lost MHC class I expression to escape $CD8^+$ T-cell responses [76]. In many HCCs, a reduced expression of ligands for activating NK-cell receptors has been observed, which correlates with increased recurrence of HCC [77, 78]. Similar to $CD8^+$ T cells, NK cells can also be suppressed by the action of IDO [79]. A direct suppression of NK cells by MDSC could also be demonstrated [80]. Finally, TAM are capable of inducing a transient activation of NK cells that results in their deletion and depends on CD48-2B4 interactions [81].

One very prominent effector mechanism of NK cells is antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC depends on the antibody-receptor CD16 on NK cells and permits them to destroy antibody-coated target cells. While there is currently no information available regarding ADCC in HCC, it is tempting to speculate about its potential, since tumor-specific antibodies have been detected in HCC patients [82]. Furthermore, a loss of $CD4^+$ follicular helper T (T_{FH}) cells that are especially important for antibody generation, is associated with reduced patient survival in HCC [83]. Thus, further research regarding the role of antibodies, B cells, and ADCC is clearly required to improve our understanding of the immune response to HCC.

Altogether, the interactions between HCC and the immune system are highly complex (Fig. 13.1). HCC engages multiple mechanisms to suppress immune responses exerted by a variety of cell types. A better understanding of these pathways and their interactions might help to establish novel, immune-based therapies to improve the outcome of patients with HCC. Based on the role of the immune system, the final two sections will discuss potential avenues for immunotherapy in HCC - on their own and in combination with current standard therapies.

13.3 Potential for Immunotherapy of HCC

Many immune-based mechanisms contribute to hepatocarcinogenesis but also immunosuppression in HCC. Accordingly, several approaches are currently under investigation to block them. In line with the role of some TLR in HCC, one early study could show that blocking TLR7 and 9 limits the growth of hepatoma cell lines in vitro and after transplantation into immunodeficient mice [84]. In contrast, triggering TLR7/8 successfully activated NK cells in vitro. These NK cells limited the growth of HCC xenografts after transfer into mice [85]. Thus, it will be important to analyze the respective effects of TLR blockade and activation in immunosufficient models of HCC where effects on both, tumor and immune system can be assessed. Another major player in progression of HCC is activation of STAT3. One study analyzed the inhibition of STAT3 in an immunosufficient model and surprisingly identified immune-mediated effects mediated by a reduced activity of T_{reg} in animals receiving STAT3 blockade which resulted in enhanced NK cell-mediated lysis of HCC [86].

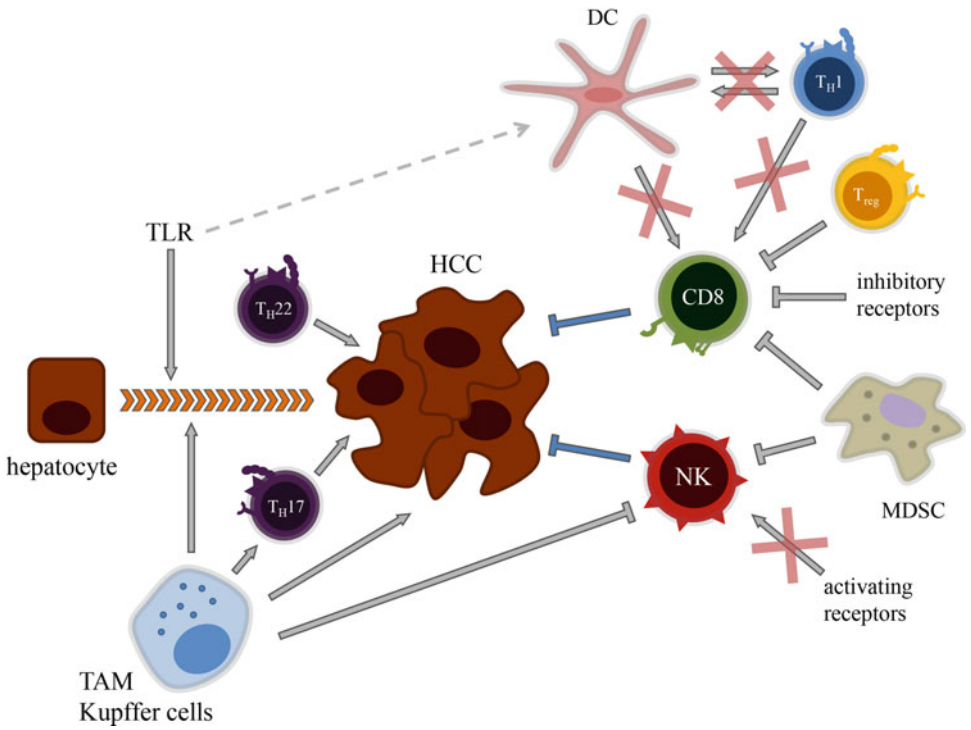

Fig. 13.1 Immune network of hepatocellular carcinoma. The malignant transformation of hepatocytes gives rise to hepatocellular carcinoma (*HCC*; *orange arrows*) and involves several components of the immune system. Toll-like receptors (*TLR*) can directly promote development and growth of HCC. However, TLR may also activate dendritic cells (*DC; dashed arrow*) and may thus have a dual role. The development of HCC is further modulated by tumor-associated macrophages (*TAM*) and Kupffer cells, as well as the activity of $CD4^+$ T helper cells producing IL-17 and/or IL-22 (T_H17, T_H22), respectively. In contrast, $CD8^+$ cytotoxic T cells and natural killer (*NK*) cells can control HCC by destruction of malignantly transformed cells (*blue arrows*). The function of $CD8^+$ T cells and NK cells in HCC patients is usually impaired by several mechanisms, as displayed. Insufficient activation of DC and type 1 $CD4^+$ T helper cells (T_H1), as well as negative signals delivered by $CD4^+$ regulatory T cells (T_{reg}), myeloid-derived suppressor cells (MDSC), and inhibitory receptors impair the function of $CD8^+$ T cells in HCC patients. MDSC and TAM/Kupffer cells also directly impair the function of NK cells, as does the decreased expression of ligands for activating NK-cell receptors in HCC

Despite the potent cytotoxicity that can be exerted by NK cells, induction of a tumor-specific $CD8^+$ T-cell response remains a major goal of immunotherapy due to the potentially lower risk of off-target toxicities. Notably, an early study demonstrated that tumor-specific $CD8^+$ T cells could be expanded from the peripheral blood of HCC patients by using autologous tumor lysate. Reinfusion of this cell product resulted in an encouraging antitumor effect, in mice as well as in five of fifteen treated patients [87]. In the meantime, several trials have confirmed that human $CD8^+$ T cells specific for AFP and other TAA can be activated in vitro by tumor-free antigen, forming the basis for vaccination trials [88, 89]. In mice, vaccination with a vaccinia virus-based vector expressing murine AFP (mAFP), resulted in generation of weak mAFP-specific T-cell responses and partial regression of established HCC lesions [90]. In HCC patients, most studies performed to date used a double vaccination consisting of a plasmid (DNA prime) followed by vaccination with an adenoviral vector (AdV boost). In this way, $CD8^+$ T-cell responses against AFP, hTERT, and multidrug resistance-associated protein 3 (MRP3) could be induced in HCC patients [91–93]. Only two patients were vaccinated against AFP. Even though both showed AFP-specific immune responses after vaccination, the tumors eventually progressed in both patients [91]. Vaccination against MRP3 and hTERT was performed in larger cohorts and induced $CD8^+$ T-cell responses in 70–75 % of patients. Tumor progression was observed in only 2/12 patients vaccinated against MRP3; however, there was no clear correlation with immunological response to vaccination [92, 93]. An earlier phase I/II study of hTERT-specific vaccination after chemotherapeutic preconditioning to lower T_{reg} frequencies, did not result in measurable immune responses against hTERT, indicating that optimization of vaccination protocols will be important to obtain optimal results [94]. While TAA-specific $CD8^+$ T-cell responses can be induced by relatively simple vaccination strategies, these appear to have a variable outcome and limited clinical benefit. Thus, more complex strategies may be required for successful immunotherapy of HCC [95].

A recent development in HCC is the direct introduction of $CD8^+$ T cells that are genetically modified to express a tumor-specific TCR into a patient with HCC. A TCR specific for AFP was identified in HCC patients and then transferred as transgene into primary human T cells [96]. These cells showed antitumor effects in vitro and in immunodeficient mice. A similar study was performed with a TCR directed against an epitope from glypican-3 [97]. Here, the authors isolated the TCR from a donor who did not naturally express the relevant MHC molecule in order to avoid a loss of highly performant TCR clonotypes by negative selection during T-cell development. However, no comparison to a "conventional" TCR was performed. Thus, it remains unclear whether the concerns regarding TCR performance were justified. Finally, a case report was published, where $CD8^+$ T cells transduced with a HBV-specific TCR were infused into a patient who had metastasized HCC expressing HBV antigens [98]. This patient had previously undergone liver transplantation and was free of circulating HBV DNA, indicating that HBV antigens were mostly expressed by metastasized tumor cells rather than hepatocytes replicating HBV. A decline of circulating HBV antigens by 90 % was observed one month after immunotherapy. However, the patient was already in a terminal stage of disease and developed brain metastases two weeks after T-cell transfer which ultimately resulted in the patient's death. Even though the results are preliminary, they show that TCR-transgenic $CD8^+$ T cells may effectively lyse target cells in terminal HCC patients, even under immunosuppressive therapy. This demonstrates the potential of TCR-transgenic $CD8^+$ T-cell therapies, if appropriate TCRs can be identified to effectively target the tumor.

Much research has been focused on using in vitro generated autologous DCs to improve the generation of tumor-specific immune responses. With antigen-loaded autologous DCs, immune responses against AFP, MAGE-A1, and glypican-3 could be induced in HCC patients [99, 100]. One of the two studies also reported a stabilization of HCC growth in one of five treated patients [100]. To broaden the repertoire of antigens presented by the DCs, other studies relied on the use of tumor lysates to load DCs with antigen. These tumor lysates were either derived from autologous tumor material or a hepatoma cell line grown in vitro [101, 102]. The two studies may indicate a superiority of using autologous tumor lysate, since a disease-control rate of 67.7 % was observed in the 31 treated patients, compared to 28 % in the 25 patients treated with DCs loaded with hepatoma cell line lysate. However, the patients treated with DCs loaded with autologous tumor lysate were divided into two groups, one of which received monthly booster infusions after initial DC vaccination. This group of patients with late stage HCC had a 1-year-survival of 63.3 % compared to 10.7 % in patients not receiving booster vaccinations [101]. Thus, the apparently higher success rate of this study compared to the one using hepatoma cell line lysate may be related to differences in protocol rather than source of antigen. These encouraging studies nevertheless provide initial evidence for further trials addressing optimizations in antigen choice and treatment protocol.

Another approach for the boosting of HCC-specific immunity is the interruption of inhibitory mechanisms limiting autologous antitumor immune responses. Since T_{reg} appear to have a prominent role in HCC, several studies have addressed potential ways to limit their function. An unmasking or an increased proliferation of TAA-specific $CD8^+$ T cells after T_{reg}-depletion in vitro has been reported [47, 73]. Similarly, depletion of T_{reg} in HCC patients by treatment with low-dose cyclophosphamide resulted in an unmasking of AFP-specific $CD4^+$ T-cell responses [103]. This indicates that depletion of T_{reg} may benefit antitumor immunity. Finally, two studies suggested an increased expression of glucocorticoid-induced tumor necrosis factor receptor (GITR) by T_{reg} in HCC patients [73, 104]. Addition of the activating ligand of GITR, GITRL, decreased functionality of T_{reg} and concomitantly improved cytokine production by $CD4^+$ non-T_{reg} in vitro [104]. Targeting GITR may thus be a simple approach to modulating T_{reg} activity in HCC patients.

Finally, a novel approach that has come into focus for therapy of cancers is the blockade of inhibitory receptors on $CD8^+$ T cells by administration of specific antibodies. This approach has resulted in FDA approval for several of these drugs, termed checkpoint inhibitors, for treatment of melanoma and other tumors [105]. In HCC, different in vitro studies observed very heterogeneous effects of inhibitory receptor blockade on $CD8^+$ T-cell function. For example, blockade of PD-L1 was described to improve proliferation but not polyfunctionality of TAA-specific $CD8^+$ T cells from HCC patients in one study, whereas others did not observe any consistent changes [45, 47]. Blockade of Tim-3 was suggested to improve proliferation and cytokine production of intratumoral T cells [58]. Since polyclonal stimulation was used in this study, the observed effect may be mediated by bystander T cells that are not tumor-specific but this nevertheless warrants further investigation. Based on the expression of Tim-3 on TAM, blockade of Tim-3 may also have a distinct antitumor effect by limiting the capability of TAM to boost HCC growth, even though this remains to be tested [24]. Finally, mixed results were described for blockade of CTLA-4, which improved TAA-specific $CD8^+$ T-cell responses, but only in single cases [46].

Importantly, the effects of blocking CTLA-4 were also analyzed in a pilot study in patients with HCC and chronic HCV infection [106]. The anti-CTLA-4 antibody Tremelimumab was administered in 90-day intervals unless limited by toxicity or tumor progression. A drop in HCV viral load

was observed together with a disease-control rate of HCC reaching 76.4 %; however, the mean progression-free survival was below 7 months. Thus, while initial results are indicating biological activity of checkpoint inhibitors in HCC, additional work is still needed and ongoing to elucidate the best approaches. Several clinical trials currently analyze the effects of antibodies against PD-1/PD-L1 and CTLA-4 in patients with HCC [105]. Despite the so far limited effect of checkpoint inhibitors on $CD8^+$ T cells from HCC patients in vitro, initial results from a phase I/II study of the anti-PD-1 antibody Nivolumab demonstrated highly encouraging results. In this study, 62 % of terminal HCC patients receiving Nivolumab were still alive after one year, compared to 30 % of controls treated with Sorafenib [107]. Of note, despite the typically strong side effects of checkpoint inhibitor therapy, no special safety issues arose in chronically HBV- or HCV-infected patients, demonstrating that checkpoint blockade can be both effective and safe in patients with virus-induced HCC. Further studies testing different checkpoint inhibitors and addressing the possibilities of combining them are ongoing. Especially in light of recent findings that indicate a higher efficacy of combined checkpoint inhibition in melanoma patients, this may further enhance therapeutic success, even though safety will have to be carefully assessed in this setting [108]. Finally, first studies are on their way to analyze the use of checkpoint inhibitor treatment in an adjuvant setting in patients undergoing standard therapies [105]. We will further discuss adjuvant immunotherapy of HCC in the following section.

13.4 Role of the Immune System in Current Standard Therapies

Adjuvant immunotherapy may be a new way to increase the efficacy of current standard therapies. Since many of the currently available therapies for HCC rely on the destruction or surgical removal of tumor masses, this may also result in a concomitant reduction of the immunosuppression exerted by the tumor. This reduced immunosuppression may facilitate immunotherapy and in turn help to limit recurrence rates of currently available standard therapies. First trials of adjuvant immunotherapy were already conducted more than 20 years ago. In one important pilot study, adjuvant infusion of in vitro activated autologous lymphocytes after resection of HCC was assessed in a randomized cohort of 150 patients and resulted in a reduction of recurrences by 18 %, as well as an increase in recurrence-free patient survival [109]. A smaller, nonrandomized study recently utilized a similar protocol extended by the injection of autologous DCs pulsed with autologous tumor lysate in addition to lymphocytes. The authors reported encouraging increases in patient survival and time to recurrence [110].

Similar studies were also conducted in HCC patients undergoing radiofrequency ablation (RFA). A recently published phase III study applying an immunotherapy protocol relying on activated autologous lymphocytes demonstrated an increase of recurrence-free survival by almost 50 % compared to controls [111]. Indeed, RFA may act as immunotherapy on its own. The necrotic cell death induced by RFA may trigger inflammation and the release of TAA [112]. Extracts from RFA-treated HCC lesions can augment the function of APC [113]. This has also been observed *in vivo* as a transient increase of DC activation in HCC patients after RFA and can result in activation of tumor-specific $CD8^+$ T cell responses [114, 115]. Furthermore, activation of NK cells after RFA was also observed [116, 117]. Thus, RFA is already associated with immune activation and further activation of the immune response appears beneficial. Less data exists for patients undergoing transarterial chemoembolization (TACE). However, two studies demonstrated an increase of tumor-specific $CD8^+$ T-cell responses in patients undergoing TACE [47, 118]. An expansion of effector-like $CD8^+$ T cells after TACE has also been reported [119]. Similar to results obtained in the setting of RFA, infusion of DC during TACE could improve recurrence-free survival of HCC patients [120].

As discussed, NK cells occur at high frequency in the liver and can have strong cytotoxic effects. One study thus investigated, whether it might be feasible to obtain NK cells from donor liver perfusates to apply them as adjuvant to prevent recurrence after liver transplantation [75]. A human NK-cell product generated in this way showed significant antihepatoma cell line cytotoxicity in vitro while not attacking partially MHC-mismatched, healthy cells derived from the transplant recipients. However, more research regarding the safety of this approach is required before it may enter the clinical setting of heterologous liver transplantation for HCC.

Finally, immunomodulatory roles have also been suggested for Sorafenib. This data has been generated in murine models of HCC, where Sorafenib inhibited T_{reg}, boosted NK-cell responses, and normalized the phenotype of TAM [121–123]. One study analyzed the role of Sorafenib on $CD4^+$ T cells [124]. Here, Sorafenib was shown to boost effector T-cell responses and to inhibit T_{reg}, but only at subtherapeutic doses. At therapeutic levels, Sorafenib inhibited effector $CD4^+$ T cells. Nevertheless, low-dose Sorafenib in combination with other therapies such as RFA or TACE may be an interesting approach to boost the immune response in HCC patients, since Sorafenib is readily available and already approved for HCC. Finally, Sorafenib may be combined with other immunotherapeutic approaches to yield synergistic effects. As recently demonstrated in a murine model of Sorafenib-treated HCC, co-blockade of C-X-C-motif chemokine receptor 4 (CXCR4) and the

inhibitory receptor PD-1 by a small molecule inhibitor and an antibody, respectively, resulted in marked inhibition of tumor growth [125]. In this model, CXCR4 inhibition limited the recruitment of immunosuppressive cells into the tumor microenvironment and thus supported the immunoactivating effect of PD-1 blockade as well as Sorafenib.

As summarized in this chapter, immunotherapy holds promise to augment the efficacy of currently available therapies, especially by lowering recurrence rates. This might reveal synergistic effects since several currently available therapeutic regimens for HCC already boost antitumor immunity and might thus help to overcome the immunosuppressive capacities intrinsic to HCC.

Taken together, the immune system has a major role in both, the development but also the control of HCC (Fig. 13.1). Multiple pathways are involved in these processes, resulting in a high complexity that may open multiple avenues for therapeutic intervention. While complex immunotherapeutic procedures relying on generation of autologous cell products have demonstrated promising antitumor effects, their complexity and cost limits their application. Simpler vaccination strategies have been met with only limited success, possibly due to immunoinhibitory mechanisms. Notably, checkpoint inhibitors are both easy to apply and very promising for therapy of HCC. Checkpoint inhibitors may be used as monotherapy as well as adjuvant therapy to support current standard therapies. This might lead to synergistic effects since several standard therapies have been shown to possess immunostimulatory effects. However, the effects of checkpoint inhibitors could so far not be readily reproduced in in vitro systems using patient-derived material. Clearly, immunotherapy is a promising new technique that has the potential to greatly enhance therapeutic options available for patients with HCC but further research is required to better understand its mechanisms of action.

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Inter- and Intratumor Heterogeneity in Hepatocellular Carcinoma

14

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14.1 Introduction

Cancer heterogeneity is an important attribute and a clinically significant factor of tumor progression. At the same time, cancer heterogeneity represents a real medical challenge because it plays a crucial role in tumor invasion, metastasis, and recurrence, and influences the effectiveness of targeted treatment and chemo- and radiotherapy.

The problem of cancer heterogeneity has become more evident in recent years due to significant progress in next-generation sequencing and single-cell analysis, which has yielded an abundant amount of data regarding the genetic complexity of tumors. However, the first mentions of the heterogeneity of cancer were presented at the dawn of cancer research. The vast heterogeneity within tumors was detected by "the first tumor pathologist," Johannes Muller, who first applied microscopy to human tumor samples in 1833. His assistant, Rudolf Virchow, described "intratumoral pleomorphism of cancer cells" and Virchow's assistant, David von Hansemann, found that "many cases of tumor have different appearances in different areas (intra-tumoural morphological heterogeneity" and showed "the degree of manifestation (i.e., de-differentiation) of an underlying tumorous process (i.e., anaplasia)… can in fact vary from area to area in either the original tumor or the metastasis" [1]. In 1914, Theodor Boveri, in his book, "On the Problem of The Origin of Malignant Tumors," reported that phenotypic differences of tumor cells can be related to chromosomal aberrations [2]. In the 1950s, histological grading in breast cancer prognosis was suggested based on the assessment of tumor heterogeneity, including the degree of structural differentiation, variations in size, the shape

and staining of the nuclei, and the frequency of hyperchromatic and mitotic figures [3]. Almost 40 years later, Nowell [4] published a key manuscript highlighting a core conception of cancer research: "Tumor initiation occurs by an induced change in a single previously normal cell which makes it 'neoplastic' and provides it with a selective growth advantage over adjacent normal cells. Over time, there is sequential selection by an evolutionary process of sublines which are increasingly abnormal, both genetically and biologically."

Currently, it is well known that the majority of cancers, including hepatocellular carcinoma, show both intertumor (interpatient) and intratumor heterogeneity, while tumor cells not only clonally evolve from a single cell of origin to more "fit" cells, but also exhibit branched evolution (like the Darwinian "evolution tree"), generating multiple distinct subclones within tumors [5, 6].

14.2 Intertumor Heterogeneity

Intertumor heterogeneity is recognized as being any type of biological difference that categorizes tumors of different patients into different subtypes with specific biological behaviors and, as a consequence, clinical treatment courses [5]. The mechanisms of intertumor heterogeneity have been hypothesized to involve either different genetic or epigenetic mutations occurring within the same cell of origin and resulting in different tumor phenotypes and/or different tumor subtypes arising from distinct cells within the tissue [7]. However, the factors and causes underlying intertumor heterogeneity are most likely identical to the ones involved in the origin of intratumor diversity and are reviewed below.

14.3 Intratumor Heterogeneity

14.3.1 Definition of Intratumor Heterogeneity

Intratumor heterogeneity is represented by any type of biological difference between tumors of the same origin or tumor cells within the same tumor in individual patients [5]. Importantly, the term "intratumor heterogeneity" can be applied to designate the diversity of endothelial, stromal, and inflammatory cells in the tumor microenvironment. Approximately, 10 biological properties underlie inter- or intratumor heterogeneity and are summarized as follows: activation of signaling pathways, evasion of antitumor immunity, induction of senescence, production of secreted factors, migration, metastasis, angiogenic capacity, genetic makeup, response to anticancer agents, and activation of metabolic pathways [8]. In fact, tumor cells within one tumor or different tumors can exhibit varying degrees of differences in regard to each of these features [8].

To date, it is well established that intratumor heterogeneity is represented by the presence of distinct cell populations, which can occupy specific microenvironmental niches, behave as communities, and substantially interact with each other and cells of the tumor microenvironment [9]. Such interactions can be both negative (from competition to cannibalism) and positive (mutualism, synergism etc.) and can significantly influence cancer progression and therapy efficiency [9, 10]. For example, the subspecialization of tumor cell functions was suggested to support cancer invasion and metastasis [9, 11], whereas cell cannibalism may be a survival strategy for tumor cells or metastases in unfavorable microenvironmental conditions [12, 13]. Intratumor heterogeneity varies dynamically throughout the disease course and has a tendency to increase as the tumor grows [6, 14]. In particular, the tumor population structure can be modulated by chemotherapy, which can either completely eradicate the tumor cell population or eliminate drug-sensitive tumor cells providing conditions for the development of drug-tolerant cells or change the tumor phenotype [15–17].

Thus, a heterogeneous tumor is now considered a dynamic and evolving ecosystem with a population structure, which varies based on changes in the selective pressure of the microenvironment, the immune system, hypoxia, therapy, etc. [5, 9, 18].

14.3.2 Types of Intratumor Heterogeneity

Intratumor heterogeneity may exist between different geographical regions of the same tumor (spatial heterogeneity) and between the primary tumor and a subsequent local or distant recurrence in the same patient (temporal heterogeneity) [19]. Spatial heterogeneity results from the fact that there are distinct microenvironmental niches in the primary tumor providing resources for the independent evolution of tumor subclones [14]. This notion is supported by the data of Gerlinger and coauthors [20] and contradicts a recent study indicating the possibility of the intermixing of tumor cells within tumor tissue [16].

Depending on the nature of the differences between tumor cells, intratumor heterogeneity can be conditionally classified as one of three types: genetic, epigenetic, and phenotypic. A major cause of genetic heterogeneity is genomic instability, resulting in an increased mutation rate and the generation of diverse tumor cell populations with specific gene mutations and chromosomal abnormalities [14, 21]. Different factors and mechanisms have been found to contribute to the origin of genomic instability, which are comprehensively reviewed in Gerashchenko et al. [5]. Epigenetic heterogeneity appears due to changes in chromatin structure (DNA methylation and histone modification), the expression

patterns of noncoding regulatory RNAs, and the deregulation of cellular network dynamics [22]. These alterations can result in the formation of distinct tumor cells with unique epigenetic profiles. Importantly, genetic and epigenetic heterogeneities mutually contribute to the clonal evolution of cancer. Epigenetic changes allow populations of tumor cells to dynamically modify networks in the same mutation pool and switch cellular phenotypes [23]. Modifications in the epigenetic landscape can promote or suppress various genetic alterations originating in the process of tumor evolution [23]. Phenotypic heterogeneity arises among tumor cells, which differ in size, shape, receptor status, differentiation, invasion patterns, etc. Typically, phenotypic heterogeneity occurs as a result of genetic and epigenetic alterations [24], although there are data that show that variations in phenotypes are not always related to gene and/or chromosomal mutations [25, 26]. In addition, tumor cell phenotype depends on the microenvironmental differences in tumors and seems to be plastic and reversible, which is confirmed, for example, by the transition between epithelial and mesenchymal states in tumor cells [24, 27].

14.3.3 The Mechanisms of Intratumor Heterogeneity

Variations in the environmental landscape, the distinct availability of resources within a tumor, and tumor-tumor and tumor–microenvironment interactions can be driving forces that generate intratumor diversity. At least five hypotheses were suggested to model the origin of heterogeneity within tumors [28], two of which, the hypothesis of cancer stem cells (CSC) and the hypothesis of clonal evolution, were described in detail. According to the CSC hypothesis, also known as the hierarchical hypothesis, tumor growth is similar to a normal physiological process, such as the development or repair of a tissue, but is initiated by genetic alterations in the stem cell. Such CSCs have increased proliferative potential and the capability to asymmetrically divide, resulting in the generation of stem cells as well as different tumor subclones [5, 29]. The model of clonal evolution, as mentioned above, was first proposed by Nowell in 1976 [4]. According to this model, tumors can arise from one cell, and cancer progression is caused by an increase in genomic instability as well as the appearance and survival of more aggressive clones under conditions of selective pressure. The clonal evolution suggests that tumor origin and intratumor heterogeneity are determined by genetic changes in somatic cells. According to this theory, the appearance of a functionally significant mutation ("driver" mutation) is favorable for divergence of the tumor clone and the generation of an evolutionarily new subclone. The intratumor genetic heterogeneity occurs due to "passenger" mutations, which are believed to be stochastic and possibly neutral or negative [5, 30]. Tumor mutations can also be classified as "background" (ancestral) or "foreground" (polymorphic). Background mutations are shared by all tumor cells in a tumor, likely to maintain cell proliferation and increase the mutation rate. Foreground mutations are responsible for the generation of tumor subclones, present only in these tumor cells, and may be involved in the transition from the least proliferative to the more aggressively growing cells [31, 32]. Background and foreground mutations can act as drivers or passengers, depending on the mutation's functional effect and penetrance.

14.3.4 Somatic Mosaicism as a Cause of Intratumor Heterogeneity

Previous hypotheses considered the development of intratumor heterogeneity within the context of the monoclonal evolution of cancer. However, if tumors are of polyclonal origin, for example originating from "field cancerization" [33], the initial heterogeneity of (normal) somatic or stem cells may contribute to the intratumor diversity. The phenomena of genetic, epigenetic, and phenotypic heterogeneity of normal somatic cells within the same tissue have been named "somatic mosaicism" [34]. The old paradigm of the genetic identity of all somatic cells in an organism has shifted after the widespread increase in genome sequencing research. It has been found that a cell has numerous genetic differences from others in the same organism [34–36]. The sources of somatic cell variability include errors in DNA replication, incomplete and incorrect DNA repair, abnormality of chromosome structure and segregation [35, 36]. The frequency of genomic changes in somatic cells is relatively high [34] and can be of great importance in different physiological and pathological processes, including tumor growth. For example, individuals with increased numbers of mosaic events were found to demonstrate an increased risk of developing cancer, while high levels of mosaicism in apparently normal matched tissues were linked to poorer prognosis in cancer patients [36]. Moreover, it is probable that mutations arise in non-malignant cells, while tumor cells merely inherit these properties. Another relation of somatic mosaicism to tumors may be the fact that the level of genomic instability in somatic cells may also be inherited by tumor cells. Therefore, the genetic instability of tumor cells and the speed of tumor progression could correlate with the level of cell mosaicism in surrounding non-malignant tissue.

However, the study of the contribution of somatic mosaicism to tumor progression encounters challenges [36]. First of all, it is impossible to estimate the mutation status of the cell genome before malignant transformation. Nevertheless, searching for tumor "driver mutations" in normal

tissues of cancer-bearing and healthy persons may be beneficial for the understanding of tumor progression. For example, it could help to explain the phenomenon of the extraordinary malignant behavior of some tumors in their early development.

14.4 Heterogeneity in Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is a structurally complex system undergoing different molecular and phenotypic changes during tumor development [37]. These changes can be represented by gene mutations and chromosomal and microsatellite instability and can operate together with nongenetic events such as alterations in the tumor cell epigenome and tumor microenvironment [37, 38]. HCCs are genetically heterogeneous diseases with characteristic morphology, growth rates, and prognosis. The presence of considerable differences within hepatocellular tumors causes uncertainty in the use of single biopsies that do not provide complete and objective information regarding tumor genotype and phenotype. It is clear that tumor heterogeneity contributes to cancer growth, metastasis, recurrence, and response to chemotherapy. Accordingly, understanding tumor diversity and the possibility of its assessment are important steps toward improving the clinical management and treatment of HCC.

14.4.1 Intertumor Heterogeneity in HCC: Insight into Molecular Classification

HCC demonstrates extremely high intertumor heterogeneity, leading to significant differences in the spectrum of morphological, immunohistochemical, and genetic features between tumors of different individuals and, as a consequence, to the presence of distinct histological and molecular cancer subtypes with specific prognoses and therapy responses [39, 40]. For example, fourteen hepatic stem/progenitor cell markers (cytokeratins 7 and 19, EpCAM etc.) have been found to be heterogeneously expressed in different HCCs. No patient expressed all of these biomarkers, and only 17.8 % of patients displayed the simultaneous expression of more than three markers [41].

At present, it is well known that HCC is represented by different histotypes: fibrolamellar HCC, clear cell HCC, scirrhous (sclerosing), combined hepatocellular/cholangiocellular carcinoma, and sarcomatoid HCC [42]. Histological classification is the gold standard for the diagnostic histopathology of HCC and is widely used in clinical practice for adequate treatment.

With the rapid development of whole-genome and transcriptome profiling technologies, including expression microarrays and next-generation sequencing platforms, molecular profiling has become a powerful tool with which to characterize the tumor landscape; identify new therapy targets, predictive and prognostic markers; and classify tumors into biologically and clinically relevant groups. The first molecular classification of HCCs was suggested by Laurent-Puig et al. [43] in 2001, who stratified tumors into two groups according to chromosome stability status. The first group included large hepatitis B virus (HBV) negative and chromosome stabile tumors possessing mutations in the *CTNNB1* gene (encodes β-catenin) and losses on 8p. HCCs of the second group demonstrated chromosome instability and different chromosomal aberrations, among which losses on 1p, 4q, 6q, 9p, 13q, 16p, 16q, and 17p were the most common, as well as frequent mutations in *AXIN1* and *TP53* genes. This group was usually associated with HBV infection, while *TP53* mutations, 17p, 13q losses, and a high value of the fractional allelic loss index were associated with poorly differentiated tumors independently of risk factors [43]. Subsequently, there were many important studies that demonstrated different molecular stratifications of HCC [44–52], which are summarized in Table 14.1. Overall, they were successful in distinguishing recurrent and frequent subtypes, such as proliferating, β-catenin-activated, interferon-related, progenitor- and hepatocyte-like subtypes, as well as less common molecular forms such as polysomy of chromosome 7-related HCC (Fig. 14.1).

Proliferating HCCs, described previously as the second group, cluster A, Met+ group, subgroups G1–G3, "proliferation" class, subclass S2, and cluster C, were found to show overexpression of the proliferation gene cluster, activation of the PI3K/Akt signaling pathway, and chromosome instability as well as being associated with poor prognosis. Patients with this subtype display high serum levels of α-fetoprotein (AFP) and have large tumors with frequent local and vascular invasion and poor differentiation [44, 46, 48, 49, 51–53].

Beta-catenin-activated tumors are distinguished based on mutations in the *CTNNB1* gene and the activation of the Wnt/β-catenin pathway. Several reports indicate the induction of TGF-β signaling, downregulation of *CDH1* (E-cadherin) and biotic stimuli/immune response genes, and chromosome stability in HCCs in this subtype [48, 49, 51–53]. These tumors are usually related to the origin of satellite lesions and vascular invasion [48, 51]. Hoshida et al. [51] also showed that the β-catenin-activated subtype is associated with a significantly greater risk of earlier recurrence.

Interferon-related HCCs have previously been identified by Chiang et al. in 2008 [49] and Toffanin et al. in 2011 [52], who demonstrated the high expression of interferon-response related genes in these tumors. HCCs of

Table 14.1 Molecular classifications of hepatocellular carcinoma

Reference	Subtypes	Frequency (%)	Biological features		Clinical features
			Chromosome aberrations/gene mutations/gene (protein) overexpression	Other genetic features	
Laurent-Puig et al. [43]	Chromosome instability (pathway I)		Losses on 1p, 4q, 6q, 9p, 13q, etc./*AXIN1*, *TP53*/–	CIN	HBV, poor prognosis, poor differentiation[a]
	Chromosome stability (pathway II)		Losses on 8p/*CTNNB1*/–		Large tumor, non HBV infected
Lee et al. [44, 45]	A versus B	44 versus 56	–/–/proliferation, cell cycle control, ubiquitination, histone modification, antiapoptotic, hypoxia genes	CIN?	Poor overall survival
	HB versus HC		–/–/hepatoblast stem, AP-1 transcription factors and target genes, JUN and FOS signaling target genes		Poor recurrence and overall survival
Kaposi-Novak et al. [46]	Met+ versus Met−	40 versus 60	–/–/Met signaling target genes (cell motility, angiogenesis etc.)		Poor overall survival, vascular invasion, microvessel density
Katoh et al. [47]	A (A1, A2, A3) versus B (B1, B2, B3)	55 versus 45	Pronounced chromosomal alterations (gains on 1q, 6p, 8q, etc., losses on 8p, 13q, etc.)/–/–	CIN	Poor overall survival, frequent intrahepatic metastasis, high serum level of AFP, HBV
Boyault et al. [48]	G1	9	–/*AXIN1*/AKT signaling target genes	CIN	Women, young, high serum level of AFP, HBV
	G2	9	–/*TP53, AXIN1, PIC3CA*[b]/AKT signaling target genes	CIN	Hemochromatosis, HBV, local, and vascular invasion
	G3	12	–/*TP53*/cell cycle control, nucleus transport genes	*CDKN2A* methylation, CIN	
	G4	38	–/*TCF1*[b]/–		Heterogeneous group
	G5	17	–/*CTNNB1*/Wnt signaling target genes	*CDH1* downregulation biotic stimuli and immune response gene downregulation	
	G6	12		*CDH1* downregulation	Satellite nodules
Chiang et al. [49]	CTNNB1	32	–/*CTNNB1*/liver-specific genes		Tumors >3 cm
	Proliferation	31	–/–/proliferation genes	CIN	Macrovascular invasion
	Interferon	25	–/–/interferon-stimulated genes		Tumors <3 cm
	Poly 7	12	Polysomy of chr 7/-/chr 7 genes	No 8q gains *EGFR* amplification[d]	
Yamashita et al. [50]		22–26	–/–/hepatoblast stem genes, Wnt signaling	High level of VEGF[c]	Poor prognosis, young, advanced TNM stages,

(continued)

Table 14.1 (continued)

Reference	Subtypes	Frequency (%)	Biological features		Clinical features
			Chromosome aberrations/gene mutations/gene (protein) overexpression	Other genetic features	
	hepatic stemcell–like (EpCAM+AFP+)		target genes, EpCAM, AFP	CK19, c-Kit expression	portal vein invasion, higher microvessel density[c]
	Bile duct epithelium-like (EpCAM+AFP−)	15–21	–/–/stem or biliary epithelial genes, EpCAM	No expression of AFP	Good prognosis, young, early TNM stages, low portal vein invasion
	Hepatocytic progenitor-like (EpCAM−AFP+)	20–22	–/–/hepatic stem genes, AFP	No expression of EpCAM	Poor prognosis, advanced TNM stages[b]
	Mature hepatocyte–like (EpCAM−AFP−)	31–40	–/–/mature hepatocyte-specific genes	No expression of EpCAM, no expression of AFP	Intermediate prognosis, elder, early TNM stages
Hoshida et al. [51]	S1	37	–/–/Wnt signaling target genes	TGF-β activation	Earlier recurrence, vascular invasion, satellite lesions
	S2	19	–/–/MYC and AKT signaling target genes		High AFP
	S3	44	–/–/hepatocyte function-related genes		Low grade
Toffanin et al. [52]	Cluster A (the wingless-type MMTV integration site)	36	–/*CTNNB1*/–		
	Cluster B (interferon-related)	33	–/–/interferon-response related genes		Small tumors (mean diameter 2.8 cm)
	Cluster C (proliferation): C1 C2 C3	31 17 9 6	C1: –/–/AKT signaling target genes C2: –/–/AKT and Met signaling target genes C3: –/*TP53*/AKT signaling target genes	miR-26a and miR-26b downregulation (C1, C2), up-regulation of C19MC miRNA cluster (C2)	Poor survival (C1, C3), vascular invasion (C1, C3), poor differentiation (C2), high AFP (C3), large tumor size (C3)

CIN, chromosome instability; HBV, hepatitis B virus; AFP, α-fetoprotein; [a]tumors with *TP53* mutations, 17p, 13q losses, and a high value of the fractional allelic loss; [b]rare events; [c]Shan et al. [54]; [d]Keng et al. [56]

this subtype displayed a smaller size (<3 cm) compared with other molecular forms.

The progenitor-like subtype is comprised of the hepatoblast-like subtype (HB) described by Lee et al. [45] as well as the hepatic stem cell- and hepatocytic progenitor-like subtypes identified by Yamashita et al. [50]. HCCs within this subtype show an enriched expression of hepatoblast and hepatic stem cell genes and are aggressive tumors with poor prognoses. Patients with these tumors usually display advanced TNM stages, portal vein invasion, high microvessel density, and poorer survival rates [45, 50, 54]. It is probable that bile duct epithelium-like HCCs also refer to the progenitor-like subtype due to the presence of EpCAM expression, which was found to be attributed to tumor-initiating cells with stem/progenitor cell features, as well as the overexpression of stem/biliary epithelial genes (*CK7* and *CK19*) [50, 55]. Interestingly, duct epithelium-like tumors show early TNM stages, low portal vein invasion, and good prognoses [50].

The hepatocyte-like subtype has been defined by the overexpression of hepatocyte function-related genes in hepatocellular tumors and the absence of EpCAM and AFP expression, which likely indicates that they originate from mature hepatocytes. These tumors tend to be well-differentiated and correlate to early TNM stages, low venous invasion, and intermediate prognosis in comparison with other HCC subtypes [50, 51, 54].

Polysomy of chromosome 7-related HCCs are distinguished by polysomy of chromosome 7 and the concomitant overexpression of multiple genes (e.g. *EGFR*) along this chromosome [49, 56]. Most of these tumors lacked 8q gains, which are the second most frequent chromosomal alterations in HCC [49].

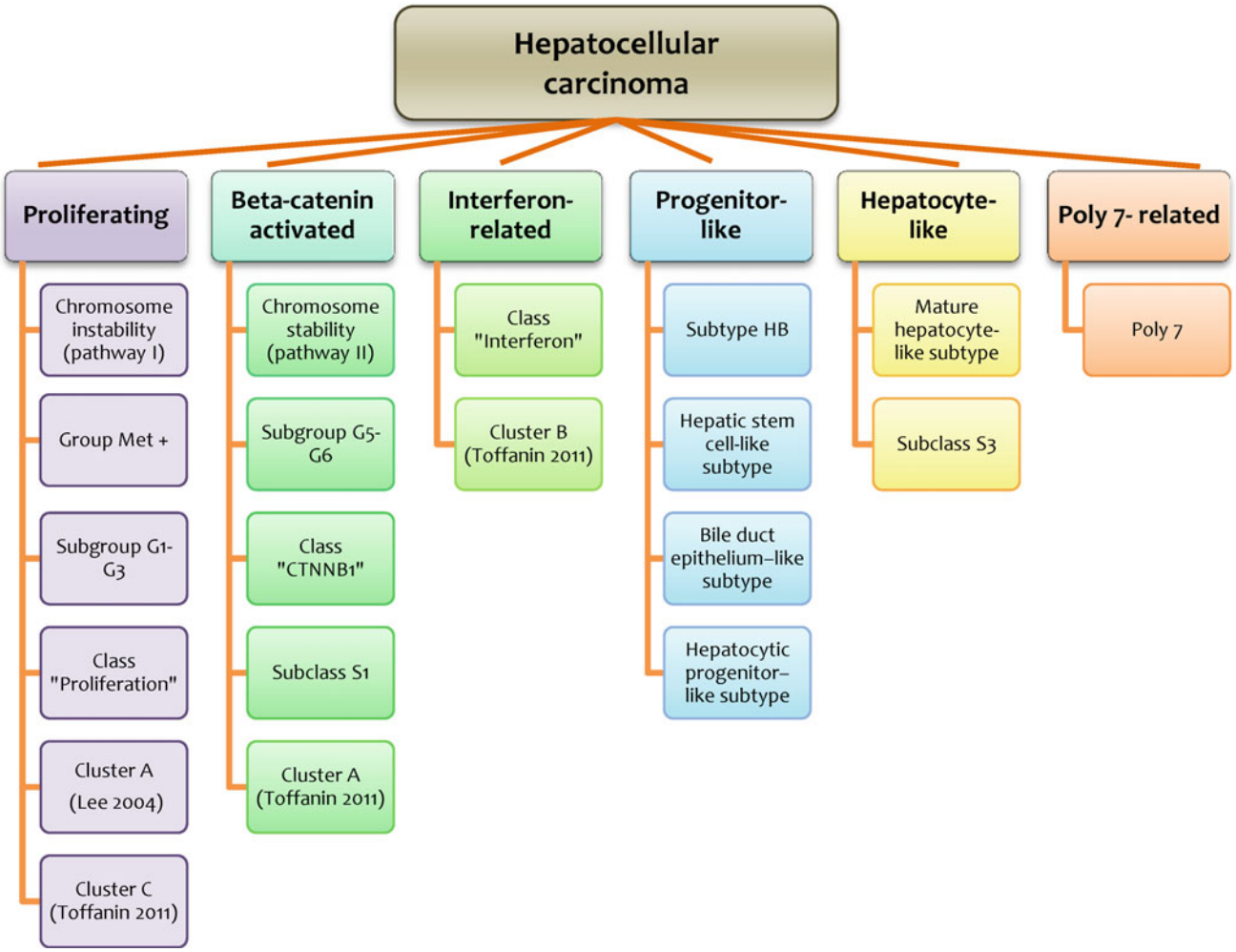

Fig. 14.1 Molecular subtypes of hepatocellular carcinoma. The recurrent subtypes: proliferating, β-catenin-activated, interferon-related, progenitor-like, hepatocyte-like, and polysomy of chromosome 7-related subtypes are given, which include previously identified HCC groups, subgroups, clusters, etc.

Recently, Zucman-Rossi et al. [57] suggested dividing HCCs into two major molecular classes. The proliferation class is characterized by an expression profile related to cell proliferation and cell-cycle control and is generally associated with more aggressive disease. Proliferating and progenitor-like subtypes likely make up this class. The non-proliferation class generally retains molecular features resembling normal hepatic physiology and includes the β-catenin-activated, interferon-related, hepatocyte-like and polysomy of chromosome 7-related subtypes.

Interestingly, intertumor molecular heterogeneity is common not only for HCC in general but also for specific histological types. For example, Cornella et al. recently suggested three different molecular classes of fibrolamellar HCC, which is a rare primary hepatic cancer that is often seen in younger individuals and is not associated with underlying liver diseases. The proliferation class demonstrated changes in the gene expression involved in the regulation of cell proliferation and the mTOR signaling pathway. The inflammation class included tumors with an altered expression of genes that regulate inflammation and cytokine production. The third "unannotated" class was characterized by a gene expression profile not previously associated with liver tumors. Surprisingly, these molecular classes were not related to survival [58].

Thus, despite a dramatic intertumor genetic heterogeneity of HCC, several prognostically distinct molecular subtypes have been distinguished that likely require specific therapeutic interventions. At present, there are many drugs that target specific molecules and are successfully applied for the treatment of different cancers. These drugs can likely be effective in the treatment of HCC because the signaling pathways involved in the development of other cancers are often seen in liver carcinogenesis (Fig. 14.2).

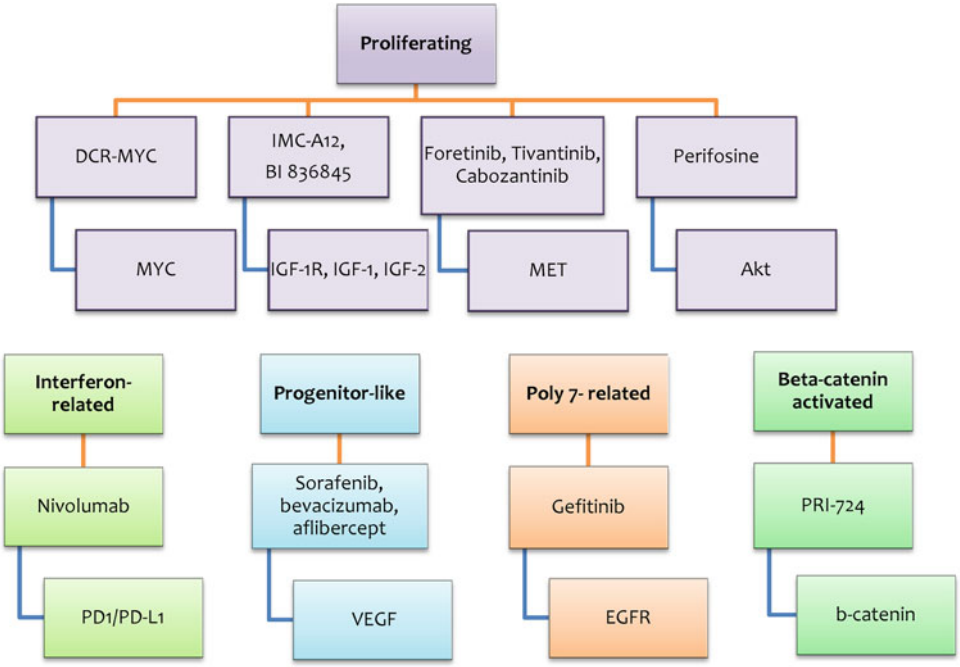

Fig. 14.2 Potential targets and therapeutic drugs in molecular subtypes of hepatocellular carcinoma

14.4.2 Intratumor Genetic Heterogeneity in HCC: Insight into Cancer Evolution

Significant genetic diversity within hepatocellular tumors was reported as early as the 1980s and 1990s. Kuo et al. [59] showed that DNA distribution patterns within a tumor were different only in 12 % of HCC patients. In the case of multiple HCCs, different DNA profiles in two distinct tumors of the same patient were evident in 29 % of cases. Interestingly, polyploid cells were more often detected in metastatic and recurrent lesions than in the corresponding primary HCCs. Another study demonstrated a heterogeneity of DNA content in 46 % of multiply synchronous HCCs, which may indicate their different clonal origin [60]. Multiclonal (polyclonal) development of multiple HCCs has also been confirmed by Sirivatanauksorn et al. [61], who described the polymorphic genomic heterogeneity between different nodules within same tumor and suggested that each nodule can be distinguished by a specific DNA fingerprint. Intratumor heterogeneity was also observed for mutations of *TP53* and *CTNNB1* genes [62–64], which are well known to be highly mutated in HCC (Fig. 14.3c).

As with others cancers, HCC is a disease of the genome. Different recurrent genetic alterations affecting tumor suppressor genes and oncogenes (*TP53*, *CTNNB1*, *TERT* etc.), as well as chromosome regions; gains at 5p, 8q, and 11q and losses at 13q and 17p are involved in hepatocellular carcinogenesis [39, 57, 65] and can act as driver mutations by either transforming normal hepatocytes into ones with malignant potential or contributing to the generation of new tumor subclones with selective advantages. Twenty-five years ago, it was suggested that a minimum of five genetic alterations are required for normal cells to become truly cancerous [66]. Quite recently, Tao et al. [31] traced the tumor evolution in one case with HCC. The whole-genome sequencing of six different samples from the primary tumor, two recurrent samples and seven specimens of tumor-adjacent tissue led to the identification of three foreground mutations, two of which affected the *CCNG1* and *p62* genes and one created an indel/fusion gene in chromosome 5q. Foreground mutations were responsible for divergent cell lineages and resulted in the origin of new tumors and recurrent subclones. In total, more than 90 % of point mutations identified here were common for different tumor regions in HCC. These mutations were classified as "background.".Twenty-four background mutations defined the common mechanisms of tumor growth, such as inflammation, cell proliferation, and migration. Thus, tumor evolution in HCC in general and intratumor genetic heterogeneity in particular have been determined to result from a long process of accumulation of background mutations and the further rapid generation of a relatively small number of foreground mutations [31]. Similar results have been previously demonstrated by Saeki and coauthors, who studied genetic differences between two regions of a single HCC nodule in six patients. They showed that while the majority of chromosomal changes were common for both

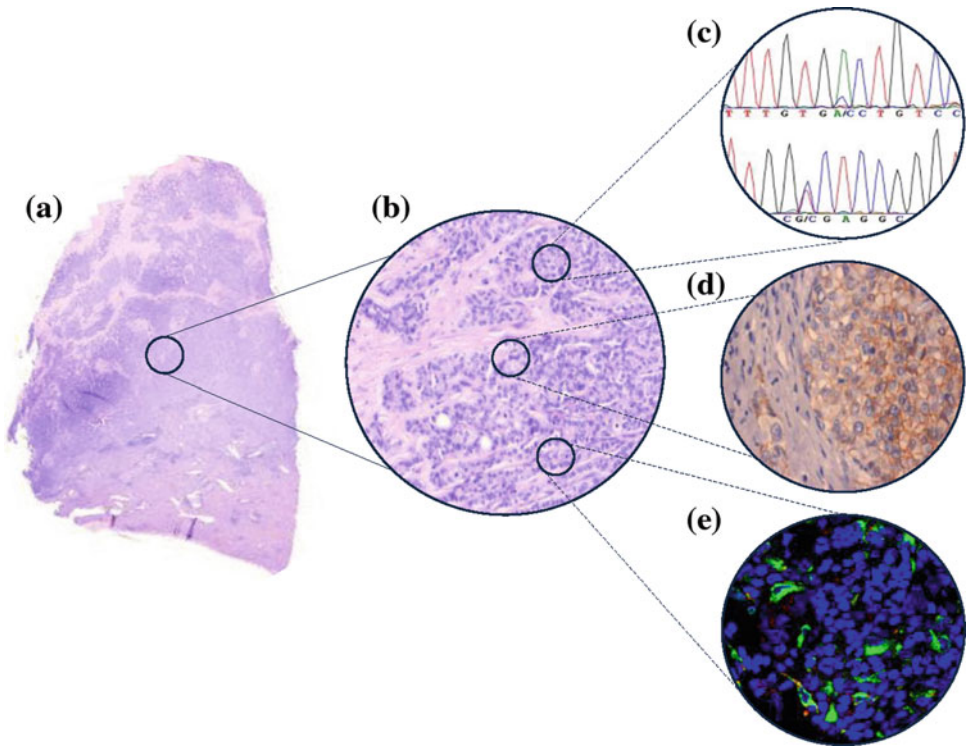

Fig. 14.3 Intratumor heterogeneity in hepatocellular carcinoma. **a** Microphotograph of hepatocellular carcinoma (hematoxylin and eosin staining). **b** Intratumor morphological heterogeneity represented by trabecular, solid, and pseudoglandular (acinar) structures in the tumor. **c** Intratumor genetic heterogeneity reflected in the variability of gene mutations (e.g. *TP53* alterations as shown in figure) across the tumor. **d** Intratumor immunohistochemical heterogeneity manifested in differences of protein expression (e.g. β-catenin) between tumor cells of the same tumor. **e** Tumor microenvironment heterogeneity arising from variations in the distribution of stromal and immune cells (e.g. macrophages) within the tumor

compartments of each HCC and likely represented a genetic basis for developing tumors, each tumor region also acquired additional independent mutations. Meanwhile, intratumor genetic heterogeneity was higher in tumor regions from advanced HCC compared to small homogeneous HCC [67].

Interestingly, the study by Tao et al. [31] did not confirm the key role of genes (*TP53*, *CTNNB1*, and *TERT*) that were previously found to be highly mutated in liver carcinogenesis [65, 68]. In particular, the frameshift mutation in the *TP53* gene detected in different tumor regions and recurrence samples was designated as a "background" mutation, whereas aberrations in the *CTNNB1* and *TERT* genes have not been identified at all [31]. These data are in agreement with the recent study conducted by Friemel et al. [37]. The authors analyzed cell and tissue morphology, the expression of the liver cell markers CK7, CD44, AFP, EpCAM, and glutamine synthetase, along with the mutations of *CTNNB1* and *TP53* in 120 tumor areas in 23 cases of HCC. The intratumor heterogeneity of at least one feature was observed in 87 % of HCC cases, whereas heterogeneity in the mutational status of *CTNNB1* and *TP53*, seen in 22 % of HCC cases, were more frequently seen in tumors with higher tumor stages (T2 and T3) and larger tumor sizes >4 cm. Importantly, *CTNNB1* mutations were not uniformly detected in all tumor regions within the same tumor. Mutations in the *TP53* gene were detected in only 2 out of 23 HCC cases, were absent in small hepatocellular tumors, and were not common within the tumor of one patient [37]. Given these results, it is possible that mutations in the *CTNNB1* and *TP53* genes represent late events in hepatocarcinogenesis and are not related to the malignant transformation of liver cells [37]. Moreover, these molecular events, particularly in the *TP53* gene, can result in clonal expansion, for example through the formation of lower differentiated tumor cells populations [62]. Moreover, intratumor heterogeneity in the mutational status of the *CTNNB1* and *TP53* genes has clinical importance because both genes are used in the molecular classification and prognostication of HCC [37, 48, 52, 53].

In addition to the above-mentioned studies, the phenomenon of clonal evolution in HCC has been demonstrated by Colombo et al. [69]. Using different approaches for cell, genome, and transcriptome analyses, as well as xenotransplantation, the authors characterized three long-term cultured cell lineages (hcc-1, hcc-2, and hcc-3) obtained from a primary hepatocellular tumor. Under identical culture

conditions, these lineages were phenotypically and genetically distinct. The hcc-1 clone contained two distinct subpopulations (clone 1/7 and 1/8) with differing growth and morphology, whereas hcc-2 and hcc-3 were identical to their mother cell populations. All of the cell populations displayed common chromosomal aberrations (translocation t(1:8) and the gain of 1q), which are most likely to be early events and involved in the origin of ancestral clones. In addition, specific chromosomal abnormalities (losses of 8p and 13q) were determined to be likely associated with tumor progression and the differing origin of these cell populations. Phylogenetic analysis confirmed the branched evolution of the oldest hcc-3 clone and the earliest hcc-2 and hcc-1 populations, including clones 1/7 and 1/8, which diverged from the ancestor of hcc-1. Clonal evolution resulted in differences in the expression profile and phenotypic drift of cell clones, which likely explains their chemoresistant and tumorigenic potentials. For example, the hcc-1 clone displayed the expression of both epithelial and mesenchymal markers, greater resistance to sunitinib, and higher tumorigenicity than hcc-3, while clone 1/7 tended to have a liver progenitor phenotype (EpCAM, CK19 etc.), and hcc-2, clone 1/8 had a phenotype of epithelial–mesenchymal (EMT) transition (Thy-1, CD105, and S100A4). Interestingly, the authors suggested that clonal cooperation between epithelial and mesenchymal cells in the hcc-1 clone is an additional factor contributing to chemoresistance. Additionally, CSCs have been hypothesized to be localized to cell lineages and to maintain/promote tumor progression and intratumor heterogeneity together with clonal evolution [69].

It is hypothesized that clonal evolution in HCC may be triggered by the HBV, which is involved in the etiology of this cancer [70]. HBV frequently integrates into the genome of liver cancer cells [71]. The consequences of such integration are quite different and usually comprise the following genetic alterations: direct gene disruption, viral promoter-driven human transcription, viral-human transcript fusion, and DNA copy number alteration [71]. Interestingly, HBV integrates in both tumor and non-cancerous hepatocytes, while clonal expansion is characteristic of only virus-integrated tumor cells [71]. Therefore, HBV integration can be used as a marker for tracing tumor evolution. Using this approach combined with genomic aberrations, Miao et al. [72] recently demonstrated the evolutionary history of multifocal HCCs in two patients and differentiated the multicentric occurrence from intrahepatic metastasis (recurrence).

Taken together, we can conclude that clonal evolution fueled by CSCs, sometimes triggered by external factors (e.g., HBV) and assisted by the accumulation of mutations with different functional effects (drivers or passengers, foreground or background), leads to the generation of genetically and phenotypically distinct subclones with independent tumor-propagating capability and differing capacities for response to therapy and, as a consequence, to the development of intratumor heterogeneity in HCC.

14.4.3 Intratumor Phenotypic Heterogeneity in HCC

HCC shows considerable intratumor morphological and immunohistochemical heterogeneity (Fig. 14.3b, d). The first is usually designated by the variability in the architectural growth patterns of individual tumor areas. In particular, trabecular, solid, and pseudoglandular (acinar) structures are accepted as distinguishing hepatocellular tumors, according to the 2009 WHO classification [73]. There is little data demonstrating that such morphological heterogeneity can be associated with larger tumor size and higher tumor stage [37]. Immunohistochemical heterogeneity is comprised of differences in protein expression (e.g., receptor status) between tumor cells within the same tumor.

The significance of morphological heterogeneity results from the fact that highly invasive tumor cells do frequently have three types of structures: trabecular, solid, and alveolar [74]. In addition, such morphological as well as immunohistochemical diversities in HCC usually correlate to genetic changes in tumor cells. For example, there is data that shows that *TP53* mutations are more often detected in the tumor cells of trabecular structures of patients from Mozambique than in cases from South Africa [75]. Pseudoglandular structures are frequently seen in tumor areas containing mutations in the *CTNNB1* gene [37]. One of the striking manifestations of morphological heterogeneity in HCC is the observation of tumor areas with two or more histological differentiation grades within one tumor in almost half of the cases. Such heterogeneity was more considerable in tumors with sizes between 3 and 5 cm than in smaller tumors (<2 cm) [76]. Subsequently, intratumor heterogeneity with respect to the histological grade has been reported in 26.8 % of cases with small HCC (<3 cm) [64]. In the study by Friemel et al. [37], such heterogeneity was detected in one fourth of HCC cases. Importantly, HCC not only displays structural (architectural) but also cytologic heterogeneity within a tumor. The different HCCs are distinguished depending on the cytological features, such as clear cell aspect, fatty and small cell change, pleomorphic cells, spindle cells, giant cells, and biliary differentiation. It is suggested that cytologic heterogeneity should be taken into account in the development of the molecular classification of HCC [77].

Intratumor immunohistochemical heterogeneity in HCC has been demonstrated in several studies and actually indicates the phenotype and the state of tumor cells in different tumor areas. For instance, EpCAM, a well-known prognostic marker of HCC, was found to be expressed in ductular reaction hepatobiliary cells within noninvasive nodules but

to be inactive in the cells located in invasive tumor regions. Furthermore, EpCAM expression in ductular reaction hepatobiliary cells was related to a higher overall survival rate and lower early recurrence rate [78]. EpCAM+ cells are also seen more frequently in association with HBV [79]. A heterogeneous expression of EpCAM in HCC tissues may be attributed to the presence of CSCs arising from normal liver stem/progenitor cells, which have various names, such as "hepatobiliary cells," "ductular hepatocytes," "atypical ductular proliferation," and "oval cells" (described in rodents) [55, 80, 81]. Increased osteopontin expression has been shown to be related to HCC metastasis [82], while osteopontin-positive tumor cells are often localized to the periphery of HCC nodules, which likely indicates the more active interaction of these cells with the tumor stroma [83]. B-catenin, which is an important player in hepatocellular carcinogenesis, was found to be heterogeneously expressed in tumor regions containing various histological grades and also in tumor regions with the same grade [64]. Other key markers (AFP, glutamine synthetase, lysyl oxidase, etc.) related to HCC growth, invasion, and metastasis have been found to be heterogeneously expressed in tumors [84–87].

In the literature, intratumor heterogeneity has mainly been described for HCC in general without referring to the histological type of tumor. Meanwhile, the presence of different histotypes of HCC is a striking manifestation of intertumor heterogeneity, which is related to cancer prognosis. Therefore, it is advisable to specify the histotype of HCC for which intratumor heterogeneity is investigated. In the available literature, there are few similar studies. For example, scirrhous HCC was shown to demonstrate considerable intratumor morphological and immunohistochemical heterogeneity. This type of HCC contained a subpopulation of small tumor cells with stem cell features, located at the periphery of tumor cell nests. Tumor cells in the central parts of tumor nests were phenotypically heterogeneous, showing variable expression of hepatocyte-specific antigen HepPar1, neural cell adhesion molecule (NCAM), and CK7 [88].

Importantly, the same hepatocellular tumor can simultaneously display variability in the genotype and phenotype of tumor cells. According to a recent study by Friemel et al. [37], overall intratumor heterogeneity was detectable in 87 % of HCC cases. However, morphological variations were characteristic in only 26 % of patients, combined morphological and immunohistochemical diversity in 39 %, and simultaneous heterogeneity of morphological, immunohistochemical, and genetic features in 22 %.

Similar to morphological heterogeneity, immunohistochemical variations within a tumor can reflect different genetic alterations and/or dynamic and reversible phenotypic tumor cell plasticity without any changes in genotype [37]. Moreover, intratumor heterogeneity in general likely provides evidence of the presence of distinct tumor cell populations within the same tumor, which complicates the classification, prognostication, and successful therapy of HCC. For instance, Villanueva et al. [89] demonstrated that gene expression signatures between the center and periphery of the tumors differed in 20–27 % of HCC patients. In this case, the use of multiple biopsies or the development of approaches for the quantitative assessment of intratumor heterogeneity could acquire more complete information regarding the tumor genetic and phenotypic landscape and prescribe the correct treatment.

14.4.4 Somatic Mosaicism as a Source of Intratumor Heterogeneity in HCC

The contribution of somatic mosaicism to the development of HCC has rarely been studied. However, there is some information on the heterogeneity of hepatocytes in normal livers and its relationship with cancer development. It is known that the livers of humans and some laboratory rodents (mice, rats) have foci of cells with unusual phenotypes [42, 90–92]. Hepatocytes of these foci have abnormal hyaline-granular, basophilic, eosinophilic, or clear cytoplasm, observed in the routine staining of histological specimens. The foci of abnormal cells have a distinct border with the surrounding liver tissue. Such foci are remarkably frequently observed in individuals with an inherited susceptibility to HCC or in animals treated with mutagenic substances or hepatocarcinogens [90–96]. Notably, the cells in the clear foci are morphologically and histochemically similar to the cells of clear cell HCC [42, 90–92]. Therefore, it may be presumed that the origin of clear cell HCC is related to abnormal foci in normal livers. Another unusual feature of cells in abnormal (basophilic) hepatocyte foci located in the portal liver lobule region is invasive growth through the portal vessel wall without metastasis [90]. Additionally, the intravascular invasion of noncancerous cells likely indicates the activation of genes, resulting in the aggressive behavior of cancerous hepatocytes. A notable example of somatic heterogeneity has been demonstrated in the enzymatic activity in hepatocytes from preneoplastic foci. Estadella et al. studied the patterns of three enzymes—glucose-6-phosphatase, adenosine-5'-triphosphatase, and 5'-nucleotidase—in 1746 hyperplastic foci (hepatocyte islands) during liver carcinogenesis induced by diethylnitrosamine in combination with phenobarbital. The authors found a clear trend toward the faster growth of cells with more deviated enzyme patterns compared with less deviated clones [97]. In addition, somatic mosaicism was found in normal liver tissues adjacent to hepatocellular tumors. A third of HCC patients have been shown to demonstrate a loss of heterozygosity or somatic mosaicism in the d(CA) dinucleotide patterns in the Cyr61

promoter in either tumors, tumor-adjacent normal liver tissues or both [96]. Cyr61 (CCN1) is a secreted protein that mediates diverse functions, including extracellular matrix formation, differentiation, cell proliferation, adhesion, migration, and survival, as well as angiogenesis and tumorigenesis [96]. Finally, mosaicism in liver tissue was described in some noncancerous diseases [98, 99]. Unfortunately, identification of other phenotypic or genetic variations in noncancerous liver cells is much more difficult. Consequently, the role of many hepatocytic properties in cancer development has not yet been studied.

Somatic mosaicism might influence the carcinogenic effects of viral hepatitis B and C infection to some extent. If individuals have various somatic mutations in susceptibility genes for hepatitis viruses, the probability of liver cancer development (especially of its multifocal forms) and polyclonality of malignant tissue would also be different, because hepatitis viruses are mutagenic agents [71, 100] and might increase the genetic heterogeneity and hepatocyte mosaicism.

Taken together, although there is not much data concerning the role of somatic mosaicism in the development of HCC and the origin of intratumor heterogeneity, it seems to be a prospective area for future studies.

14.4.5 Tumor Microenvironment Heterogeneity in HCC: The Role in Cancer Progression

HCC is a typical inflammation-related cancer characterized by the close relationship between the tumor microenvironment and tumor cells. Chronic low-grade inflammation influences both innate and adaptive immune responses, resulting in a tolerogenic environment, which leads to tumorigenesis and further tumor progression [101]. In the majority of cases, HCC develops after persistent chronic liver diseases caused by hepatitis B or C infections. These viruses induce chronic inflammation, which may result in the malignant transformation together direct oncogenic virological activity [102]. In particular, viruses play a crucial role in modulating the accumulation and activation of both cellular components (immune cells and fibroblasts) and noncellular components (cytokines and growth factors) of the microenvironment, markedly influencing disease progression and prognosis [103]. Thus, the immune system is very involved in HCC pathogenesis, but the intrinsic mechanisms of immune system-tumor interrelationship are not completely understood [101].

The tumor microenvironment consists of several cell types, including hepatic stellate cells (HSCs, or myofibroblast-like cells), fibroblasts, and immune (effector and regulatory lymphocytes, macrophages etc.) and endothelial cells that actively contribute to tumor initiation, progression, and dissemination. In turn, the tumor itself induces stromal cells to create microenvironmental conditions to maintain tumor growth and metastasis [101]. Different distributions of HSCs and inflammatory cells were previously observed in HCC tumors (Fig. 14.3e) and were related to cancer prognosis. For example, a high density of macrophages, activated HSCs and mast cells as well as a high expression of macrophage colony-stimulating factor/its receptor and placental growth factor, Th1/Th2-like cytokine shift, inflammation-related signature have been found to be associated with late recurrence [104].

HSCs are important players in the tumor microenvironment and are closely related to HCC prognosis. Many data support the protumor function of activated HSCs. The heterogeneous distribution of HSCs in HCC tumors is a known phenomenon. In a study by Liao et al. immunohistochemical analysis showed various distributions and expression intensities of the most prominent HSCs markers, including α-SMA, glial fibrillary acidic protein (GFAP), desmin, vinculin, and vimentin, which likely result in the different biological behaviors of these cells and the cellular responses to injurious stimuli in HCC progression. In particular, it has been demonstrated that peritumoral-activated HSCs were poor prognostic factors for resected HBV-related HCC, especially in the early-recurrence and AFP-normal subgroups. Moreover, researchers have shown for the first time the expression of fibrogenesis- and hepatocarcinogenesis-related genes in peritumoral HSCs. It is most likely that all of these changes in the HSC phenotype reflect different cell states and are potential targets of HCC therapy [105].

Tumor infiltrating lymphocytes (TIL) are part of the tumor surveillance system. TILs not only function as part of the defense system, but also as regulators of immune tolerance. Tumor infiltration by lymphocytes has been demonstrated to vary in different regions of tumor tissue. In HCC, lymphocytes were found to be localized around the tumor, while CD4+ T cells such as helper or regulatory cells were concentrated in the peritumoral region. Previously, it was suggested that CD4+ T cell infiltration may be a sign of tumor adaptation, known as tumor enhancement [106]. In the tumor itself, the infiltration was represented by CD8+ cells. The CD20+, TIA-1+, and CD56+ cells of the innate immune system were practically absent. Histogenetic origin (intertumor heterogeneity) did not influence the TIL patterns in tumors. Interestingly, researchers did not find any correlations between the distribution of TILs and the clinicopathological data [107]. In another study, CD4+ CD25+ T regulatory cells were shown to be more prevalent than CD8+ T cells in HCC tumors compared with adjacent benign tissue; this predominance of Treg cells is associated with a worse prognosis. Functionally, Treg cells impair cytotoxic CD8+ T cell proliferation, activation, degranulation, and the production of granzyme A, granzyme B, and perforin [108].

In addition, increasing Treg cell prevalence has been shown to strongly correlate with advancing stages of HCC progression [109].

Zhou et al. [110] investigated peritumoral and intratumoral hepatic tissues of patients with HCC after curative resection to see whether inflammatory cytokines are correlated with prognosis. It was shown that higher levels of IL-2 and IL-15 in peritumoral liver tissues, but not in tumor tissues, are significantly associated with a decreased incidence of recurrence of intrahepatic tumor and prolonged overall survival. Similarly, Zhu et al. [111] showed a significant association between peritumoral expression of macrophage colony-stimulating factor and the poor prognosis of HCC. It is important that the prognostic values of IL-2 and IL-15 did not depend on any clinicopathological factors and were confirmed for early stages of HCC, making them promising markers for disease prognosis [110].

It is known that the imbalance of Th1/Th2-like cytokines influences inflammatory conditions within the tumor, determining the malignant phenotype of HCC. Peritumoral levels of Th1/Th2-like cytokines are useful for stratifying patients, even those with early-stage HCC, into subgroups with different prognoses following curative resection [112]. For example, treatment with Th1 cytokine IFN-α after curative resection prevented early recurrence and improved the overall survival of HBV-related HCC possibly by correcting the imbalances [112, 113].

The heterogeneity in the immune microenvironment within HCC tumors and its association with disease prognosis were also described by Chew et al. In particular, proliferating immune cells, mainly NK and T cells, were present in areas without proliferating tumor cells and were linked with longer survival. NK and CD8(+) T cell densities appeared to be positively correlated with the apoptosis of tumor cells and negatively with tumor cell proliferation [114].

Tumor-associated macrophages (TAMs) are considered to promote tumor growth and metastasis. To date, there is no general agreement regarding the influence of TAMs and their numbers on HCC progression [115]. Recently, a twofold decrease in the number of TAMs (CD68+) between intratumoral and peritumoral territories in HCC was found. TAMs were predominantly seen in peritumoral areas, likely pointing to their location at the tumor invasion front. The number of TAMs was not associated with clinicopathological signs. However, the increased number of peritumoral TAMs in primary tumors was associated with better prognoses, whereas the lower number of TAMs in intratumoral areas was related to the tumor cell microenvironment [115]. The low number of TAMs in the intratumoral area was suggested to be linked to the negative effects of tumor cells, namely tumor cell-induced macrophage apoptosis [115]. The data obtained are rather difficult to analyze due to serious limitations related to the lack of macrophage subtype detection. CD68 could not distinguish between M1 and M2 subtypes. M1 macrophages activated mainly by bacterial lipopolysaccharides and immune stimuli such as interferon-g (IFN-g) have antitumor roles due to the elimination of tumor cells, antigen presentation, T cells, and the synthesis of numerous proinflammatory cytokines [116–118]. M2 macrophage differentiation results from the contact with Th2 cells or after stimulation by cytokines (e.g., interleukin (IL)-4, IL-10, IL-13) and growth factors, such as TGF-β [117, 119]. Because only the basic identification of macrophages has been performed by Avadanei et al., future studies should assess the roles of the M1 and M2 subpopulations of macrophages in HCC.

In the literature, there are several reports regarding the role of M2 macrophages in HCC pathogenesis. Yeung et al. [120] showed that all macrophage-associated receptors (CD14, CD68, CD163) were more highly expressed in the peritumoral region than in the intratumoral region, indicating elevated numbers of macrophages in the tumor peripheral area. In addition, this study provided the new insight that only peritumoral M2 macrophages significantly contribute to HCC progression. In particular, high levels of peritumoral M2 receptors (CD163 and SA) were associated with poor survival and increased rates of intrahepatic recurrence. In this case, the assessment of M2 macrophages in the peritumoral region after hepatectomy could be useful for the identification of patients with a high risk of HCC recurrence. Increased levels of peritumoral M2 macrophages were also associated with advanced tumor stages, multiple nodules, and venous infiltration, which indicate their potential roles in facilitating tumor cell dissemination and invasion. Notably, in vivo and in vitro experimental results confirmed the M2 protumor functions in HCC, showing their stimulated effect on tumor growth and migration. CCL22/CCR4 signaling has been shown to markedly contribute to enhanced HCC invasiveness due to EMT activation [120]. Thus, the clinical value of M2 macrophages as an independent prognostic indicator for poor prognosis in HCC has been demonstrated. Interestingly, researchers demonstrated that tumor cells can induce the production of CCL22 in M2 macrophages, which in turn enhanced tumor migration capacities via EMT activation. In accordance with these results, the authors suggested that CCL22-related tumor invasiveness is explained by the CCR4-assisted migration of HCC cells toward the peritumoral regions, where CCL22-producing M2 macrophages predominantly reside [120].

Wang et al. [121] presented unique data concerning the role of the microenvironment in spontaneous HCC regression. They examined a patient with spontaneous regression of HCC, as detected by histological and immunohistochemical exam, and compared this case to 20 cases of nonspecific HCC. Microscopically, the tumor was an almost

completely necrotic nodule with inflammatory cell infiltration and was encapsulated by a fibrotic capsule. Many inflammatory cells within the periphery of the tumor were found, while only a minority of them infiltrated the central zone of the tumor. CD68+ single macrophages were localized both to the central zone of surviving portion of the tumor and the fibrous capsule of the periphery of tumor. Meanwhile, the level of CD163+ cells was higher in partially surviving tumors than in the fibrous capsule and peritumoral liver tissue. It is important to note that CD163+ cells in the surviving tumors were larger in size and were likely activated, whereas small peritumoral CD163+ cells seemed to be dormant. Based on these data, the authors concluded that highly activated macrophages (CD163+) in tumors contributed to the spontaneous regression of HCC [121]. However, it is not possible to state whether HCC regression is related to M2 macrophages because, as the authors concluded [121], CD163 alone cannot differentiate between activated and inactivated macrophages, for which the involvement of additional M2 markers (e.g., stabilin-1 [122], mannose receptor [123] and some others) is necessary.

B cells are abundantly present in tumors, while the role of these cells in cancer pathogenesis remains unclear. Recently, it has been demonstrated that chemokine (C-X-C motif) receptor 3-positive (CXCR3+) B cells constitute almost half of the B-cell infiltrate in HCC and that their levels are positively correlated with the early recurrence of HCC [124]. These cells selectively accumulate at the invading front in HCC tumors, undergoing further somatic hypermutation and differentiation in plasma cells. Moreover, CXCR3+ B cells, but not their CXCR3− counterparts, may induce the polarization of regulatory macrophages (M2b) in HCC via immunoglobulin G-dependent pathways. The significant suppression of M2b polarization and the protumorigenic activity of tumor-associated macrophages were evident when B cells were abrogated. This finding points to the idea that blocking CXCR3+ B-cell migration or function may be considered to be a potential target for cancer therapy [124].

Thus, the tumor microenvironment in HCC has been described as heterogeneous. Several studies found that heterogeneity in the HCC environment is not a random event and is associated with tumor growth, invasion, and metastasis, and, as a consequence, contributes to the prognosis and survival of patients. Moreover, immune cells located predominantly in peritumoral regions and related to poor prognosis could be attractive targets for HCC therapy.

14.5 Summary

HCC has been characterized as a complex disease that demonstrates considerable inter- and intratumor heterogeneity involved in tumor growth, invasion, and metastasis. The phenomenon of intertumor heterogeneity has been well studied in terms of genetics, histology, and impact on the clinical manifestation of HCC. In particular, the histological and molecular classifications of HCCs have been suggested based on an analysis of the morphological, genomic, and molecular features of tumors. Different HCC subtypes demonstrate specific clinicopathological characteristics and have distinct potential against cancer progression. The implications of these classifications in clinical practice make it possible to predict prognosis and, as a result, to choose a better mode of treatment. However, it must be noted that the molecular classification of HCC is not adequately validated in terms of the clinical behavior of tumors and sensitivity/resistance to therapy. Meanwhile, the accurate analysis of the available results regarding the molecular mechanisms of intertumor heterogeneity and the investigation of genetic or phenotypic variability within certain histological forms of HCC could be used to develop a more adequate classification, though its effectiveness should be clinically validated.

Despite abundant data concerning the nature and mechanisms of intratumor heterogeneity in HCC, routine qualitative and quantitative criteria are not thought to accurately capture the complete genomic landscape of cancer or to assess the risk of cancer progression and predict therapy response. The use of multiple biopsies is not always possible and is associated with clinical risk, patient choice, technical and ethical problems. The most sensitive and accurate genomic technologies, such as deep sequencing, which can detect sequences or mutations occurring at very low frequencies, and, as a consequence, observe minor tumor cell populations, remain sufficiently expensive and complicated, and may miss important information regarding the genetic landscape due to inadequate sampling.

In this regard, the simplest targets for assessing intratumor heterogeneity are the morphological features of tumors, which can be easily detected by histological analysis and act as a routine method of clinical diagnosis. For example, we previously showed the presence of five types of distinct morphological structures in breast tumors, which were associated with different risks of cancer metastasis and chemoresistance [125]. In a series of studies, molecular genetic analysis was performed to clarify the possible mechanisms contributing to the biological behavior of breast tumors with a prevalence of those or other morphological structures [126, 127]. According to this view, further studies are needed to reveal effective criteria for assessing the clinical significance of intratumor morphological heterogeneity in HCC. Future research would make it possible, on the one hand, to identify pathogenetically significant alterations, which determine phenotype and behavior of morphologically distinct patterns of tumor, and, on the other hand, to validate their value for clinical practice. It should be noted that this approach may be used to establish the clinical relevance of heterogeneity in the tumor microenvironment,

whereby not only prognostic and predictive markers but also therapeutic targets may be identified both in tumor cells and stromal-inflammatory infiltrate.

Another promising method for the analysis of intratumor heterogeneity in clinical practice could be imaging, which allows one to assess the spatial variation in the architecture and function of individual tumors via the quantification of basic biophysical parameters, such as density or MRI signal relaxation rate, through measurements of blood flow and volume, hypoxia, metabolism, cell death, and other phenotypic features, as well as through the mapping of the spatial distribution of biochemical pathways and cell signaling networks [6]. Imaging can also assess the receptor status of whole tumors at multiple sites and at several time points [128]. In addition, the radiomic signature capturing of intratumor heterogeneity has been shown to be associated with cancer prognosis and underlying gene expression patterns [129]. Thus, medical imaging can identify different phenotypes and possibly distinct cell populations existing within a tumor and represents a routine method for quantifying intratumor heterogeneity in clinical diagnostics. However, future radiogenomic studies are required to establish a correlation between different genetic phenotypes of tumor cells (including prognostically important molecular changes) and radiomic signatures.

Finally, the detection of circulating tumor DNA or tumor cells in liquid biopsies can be an effective prognostic and predictive method that overcomes the problem of intratumor heterogeneity. The use of this approach allows the detection of known genetic changes and/or the elucidation of new molecular aberrations to obtain timely information regarding the origin of new clones in tumors and to choose accurate and adequate therapies. Nevertheless, some issues remain: (a) there is no comprehensive data regarding the types and numbers of driver mutations in different cancers; (b) it is unknown to which level of change in driver mutations the therapy should be modified; and (c) the degree of the heterogeneity of the targets for targeted therapy has not yet been determined.

Taken together, all available evidence indicates that further studies are needed to clarify the mechanisms and clinical significance of intratumor heterogeneity and to identify new prognostic, predictive markers, and therapeutic targets.

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Systemic Inflammation: A New Prognostic Domain and Source of Therapeutic Targets in Hepatocellular Carcinoma

15

David J. Pinato and Rohini Sharma

Contents

15.1 Introduction

The relationship between inflammation and cancer is embedded within the foundations of cellular pathology itself, when the German pathologist Rudolf Virchow first postulated that the immune infiltrate commonly found adjacent to most neoplastic tissues could be more than an innocent bystander but rather an active player in the tumorigenic process [1]. Hepatocellular carcinoma (HCC), is the third most lethal solid tumour worldwide [2], and represents one of the malignancies where inflammation plays a critical pathogenic role, given that at least 80 % of hepatocellular tumours arise as part of a continuum where chronic liver disease culminates into malignant transformation through liver fibrosis [3].

While it is recognised that each risk factor for HCC can promote hepatocarcinogenesis through different molecular pathways, inflammation has emerged as a unifying mechanism across most aetiologies: from hepatotropic viral infection [4] to alcohol-related fibrosis [5] and non-alcoholic steatohepatitis [6]. The activation of pro-inflammatory pathways represents a substantial part of the inter-cellular cross talk between tumour cells and diverse cellular subsets including angiogenic, immune, and cancer-associated fibroblastic cells globally termed as the "tumour microenvironment" [7]. Persistent and unopposed cytokine release accompanies the transition between fibrosis to carcinoma, with a few distinct molecular mediators including interleukins (IL) and chemokines exerting a well-defined promoting role [8]. While initially focusing on the pathogenesis of HCC, more recent studies have established a clear prognostic role of inflammation and the clinical course of the disease.

Evidence from gene expression profiling studies demonstrate a Th-1 to Th-2 cytokine shift within the peritumoral tissue that can significantly affect the probability of recurrence and mortality following radical resection of HCC [9]. Similarly, a wide range of single candidate based studies illustrate that unopposed local or systemic release of

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pro-inflammatory mediators can predict for an adverse course of the disease. In the case of IL-2 and IL-15, for instance, elevated peritumoral expression predicts for early recurrence and shorter survival after resection [10], while in the case of IL-10, elevated secretion tends to associate with immune dysfunction, with impaired dendritic cell maturation [11] and an increased proportion of myeloid derived suppressor cells (MDSC), a constellation of molecular traits that links inflammation with the molecular progression of HCC [12].

Other studies have linked a pro-inflammatory tumour microenvironment to enhanced angiogenesis, such as in the case of IL-17 rich tumours, where recruitment of IL-17 producing T-helper cells can adversely influence patients' prognosis through fostering angiogenesis [13].

In aggregate, these studies have provided in-depth mechanistic insight into how inflammation can drive the progression of HCC as a result of the complex interaction between several domains of cancer biology (i.e. angiogenesis, unrestrained proliferation, immune dysregulation) in a dynamic cross-talk between the tumour itself, its immediately surrounding microenvironment and the host's immune response.

A significant number of studies have investigated the prognostic role of a number of inflammation-based signatures in HCC either by measuring inflammatory biomarkers in tumours, surrounding liver tissue or in the systemic circulation, with none however completing the transition from being a scientifically interesting trait to a clinically useful test. Barriers to tissue based and immunological approaches reside in the poor accessibility and costs involved in applying standardized genomic analysis of tissue samples. While potentially more accessible, quantification of circulating cytokines may prove a suboptimal prognostic tool for a number of reasons. Firstly, the abundance of candidates combined with the pleiotropic and redundant nature of cytokine signaling makes a combination of molecular actors rather than one single cytokine the likely driver of cancer-related inflammation. Secondly, peripheral blood cytokine measurement may not necessarily mirror the local pro-inflammatory milieu responsible of adverse clinical outcomes. Thirdly, the paucity of studies confirming the prognostic role of circulating cytokines and their improved accuracy over routinely measured prognostic markers in HCC makes it difficult to draw conclusions as to which candidate should be taken forward for routine clinical evaluation.

In recent years, research into inflammation-driven changes in routinely available peripheral blood parameters has provided further insight into cancer-related inflammation in HCC. Several studies have identified a number of inflammation-related traits in the peripheral blood of patients affected by HCC including leucocytosis [14], thrombocytosis [15], relative lymphopaenia [16], increased levels of C-reactive protein (CRP) [17], hypoalbuminaemia [18], hyperferritinaemia [19] and elevated plasma fibrinogen levels [20].

Consolidated evidence emerging from a growing number of clinical studies shows that the combination of the diverse acute phase reactants can be used to derive composite, inflammation-based prognostic scores. These include the neutrophil-to-lymphocyte (NLR) and platelet-to-lymphocyte ratio (PLR), the prognostic nutritional index (PNI), derived from a nomogram based on hypoalbuminaemia and lymphopaenia (albumin in g/dL $\times$ 10 + 0.005 $\times$ total lymphocyte count), the Prognostic Index (PI), calculated using leukocytosis (>11.000/μl) and elevated CRP (>1 mg/dL) [21] and lastly the modified Glasgow Prognostic Score (mGPS)—recently renamed as inflammation based index (IBI) in the context of HCC—which combines hypoalbuminaemia (<35 g/L) and elevated CRP (>1 mg/dL) (Table 15.1) [22].

In the last decade, the expansion of studies investigating the prognostic power of inflammation based indices in solid

Table 15.1 Computation of inflammation-based prognostic index in HCC

Inflammation based prognostic index	Score
Inflammation based index/mGPS	
CRP $\leq$ 10 mg/L	0
CRP > 10 mg/L + Albumin $\geq$ 35 g/L	1
CRP > 10 mg/L + Albumin < 35 g/L	2
GPS	
CRP $\leq$ 10 mg/L + Albumin $\geq$ 35 g/L	0
CRP > 10 mg/L + Albumin < 35 g/L	1
CRP > 10 mg/L + Albumin < 35 g/L	2
PI	
CRP $\leq$ 10 mg/L + WCC < 11.000/μL	0
CRP $\leq$ 10 mg/L + WCC > 11.000/μL	1
CRP > 10 mg/L + WCC < 11.000/μL	1
CRP > 10 mg/L + WCC > 11.000/μL	2
PNI	
Albumin (g/dL) $\times$ 10 + 0.005 $\times$ lymphocyte count $\geq$ 45	0
Albumin (g/dL) $\times$ 10 + 0.005 $\times$ lymphocyte count < 45	1
Neutrophil-to-lymphocyte ratio	
Total neutrophil count/total lymphocyte count Different cut off values used: 3:1, 5:1	0/1
Platelet-to-lymphocyte ratio Total platelet count/total lymphocyte count Different cut off values used:	
<300:1/>300:1	0/1
<150:1/150–300:1/>300:1	0/1/2

tumours has been unprecedented, with more than 60 studies having explored the prognostic value of the NLR across >37.000 patients with solid tumours [23], and similar figures applying to the evaluation of the GPS and mGPS [24]. The prognostic qualification of inflammation-based indices has more recently extended to HCC, where an increasing number of studies have assessed each biomarker either individually or in comparison both in the curative as well as in the palliative setting.

In this chapter we summarize the current body of knowledge around the use of inflammation-based indices in HCC and their positioning in the routine prognostic assessment of HCC with respect to established staging systems and current treatment algorithms. Secondly, we aim to summarize the knowledge gathered around the biologic foundations supporting the prognostic deterioration observed in patients with deranged inflammatory scores. Thirdly, we explore whether suppression of cancer-related inflammatory response may serve as a therapeutic strategy against HCC. Lastly, we discuss the criticalities surrounding optimal clinical application of inflammation-based indices in HCC and the open questions around their use.

15.2 Inflammation-Based Indices: Biologic Qualification of Their Prognostic Role in HCC

15.2.1 NLR

In the context of an acute phase response, the relative ratio between neutrophils and lymphocytes within the total white cell count changes from a normal proportion of 50–60 and 30–40 % respectively to reflect a condition of peripheral blood neutrophilia and relative lymphopenia, causing the ratio to abnormally increase above its normal value of 2.

It has been shown that granulocyte recruitment and activation is in part directly fostered by solid tumours through the activation of oncogenes such as *RAS* and *MYC* with the aim to render the tumour microenvironment a cytokine-rich background able to facilitate angiogenesis and tumour progression [25]. Mounting evidence suggests that the process of granulocyte recruitment and activation is largely cytokine-driven and reflects at least in part a paracrine and endocrine effect stemming from hypoxic and frankly necrotic tumourous tissue as part of an ongoing pro-angiogenic signaling cascade. Activation of the hypoxic response pathways with release of pro-angiogenic cytokines represents a recognized mechanism of neutrophil chemotaxis to the peritumoral stroma [26].

In addition, the progression from hypoxia to anoxia within the tumour with the emergence of necrosis may further trigger the innate immune response via the release of damage-associated molecular pattern molecules (DAMPs), activation of the complement cascade, and release of opsonins, resulting in an overall increase in the absolute neutrophil count [27]. It has been shown that local release of pro-angiogenic cytokines including IL-17 is crucial to this process in HCC [28]. More recent evidence has linked CXCL5 with neutrophil infiltration and shorter time to recurrence, confirming a prognostic role for neutrophil infiltration in dictating the natural course of the disease in a large series of resected specimens [29].

Sustained angiogenesis is not the sole mechanism underlying neutrophil-mediated tumour promotion [27]. The inflammation-driven generation of reactive oxygen species as part of the oxidative burst may have an influence on tumour progression by facilitating genomic instability. Secondly, interaction with the extracellular matrix through the release of proteases and activation of Hepatocyte Growth Factor (HGF) signaling pathways are neutrophil-facilitated strategies underlying the acquisition of an invasive phenotype [30].

While initially thought as terminally differentiated innate immune effector cells, neutrophils are now recognized to have some degree of plasticity in their response, allowing for contextual changes in the pattern of sensitivity and response to different cytokines, which adds a further layer of complexity in understanding their role in cancer-related inflammation [31]. Tumour-derived secretion of Transforming Growth Factor-β (TGF-β), a cytokine that has been implicated in the progression and metastatic dissemination of HCC [32], has emerged as a key cytokine capable of polarizing neutrophil responses from an anti-tumoural "N1" to a pro-tumoural "N2" phenotype. Such coordinated response does not happen as a result of an exclusive interaction with the tumour but is part of a more complex network of inter-cellular interactions involving other actors of the tumour microenvironment including macrophages, stromal cells, and lymphocytes. Interestingly, a recent study by Mano and coworkers revealed that HCC patients with an elevated NLR had a higher proportion of macrophage peritumoral infiltrate and worse prognosis after curative resection [33]. While the functional background behind this association was not clarified, growing evidence suggests an immunosuppressive role for tissue macrophages through various mechanisms: TGF-β overexpression [34], which promotes N2 polarization, expression of PD-1 ligand 1 (PD-L1), which suppresses the cytotoxic function of PD-1-expressing CD8+ T cells [35], or by secretion of immune-suppressive cytokines such as IL-10 [36]. Accumulating evidence suggests that the differentiation of resident tumour associated macrophages (TAMs) into an immune-regulatory, pro-tumourigenic "M2" phenotype is crucial in governing the fate of circulating immune cells including neutrophils and lymphocytes in the tumour

microenvironment [37]. In parallel, a similar immunologic network can promote the emergence of myeloid derived suppressor cells (MDSC), an immature population of innate immune cells that can influence tumour progression by inhibiting antitumour CD8+ T cell as well as NK cell responses [38].

While an increasing number of studies across a wide range of malignancies have established that the cross-talk between the local immune response and systemic inflammation is the result of a causal relationship rather than a simple epiphenomenon [39], the molecular and immunological drivers responsible for such parallelism have not been yet fully elucidated in HCC and warrant further clarification in adequately powered clinical studies.

15.2.2 PLR

During acute inflammation, reactive thrombocytosis is a systemic response aimed at facilitating the resolution of tissue injury by promoting local haemostasis and wound healing through the focal release of a wide range of platelet-derived humoral signals. There is evidence to suggest that in cancer, such response is subverted by the presence of systemic cytokine release that acts on platelets to achieve an autostimulatory loop and increase platelet-secreted mediators such as Platelet-derived growth factor (PDGF), Vascular endothelial growth factor (VEGF) and others [40].

In the context of HCC, platelet counts are influenced by the presence of underlying cirrhosis, which, in a significant proportion of patients, induces thrombocytopaenia through hypersplenism secondary to portal hypertension [41]. A number of studies have combined absolute platelet counts with other biomarkers of liver function to generate combined prognostic scores such as for instance the aspartate aminotransferase (AST)-to-platelet ratio index (APRI). Increasing evidence suggests an independent prognostic role for APRI in defining the risk of HCC recurrence after primary ablation or resection, especially in hepatitis B virus (HBV)-related HCC [42–44].

More recent studies however highlight a correlation between thrombocytosis and adverse clinico-pathological features suggesting platelet count may also identify a more aggressive neoplastic phenotype, independent of liver function [41, 45]. Mechanistic studies have shown that platelet lysates can promote tumour cell proliferation [46] and antagonize the effects of sorafenib-mediated cytotoxicity in vitro, suggesting that platelet activation may oppose treatment efficacy in patients receiving systemic treatment for HCC [47]. Further research has shown that platelets may exert a more complex immunopathologic role in HCC, modulating the hepatic accumulation of virus-specific CD8+ lymphocytes and enhancing local necro-inflammatory damage, which in turn predisposes to the onset and progression of HCC [48].

An adverse prognostic role for inflammation-driven reactive thrombocytosis has been described in HCC, with a strong correlation observed with infiltrative pattern of growth [49]. While the PLR seems to hold inferior prognostic ability compared to other inflammation based scores in HCC [22, 50], recently published evidence has shown that combination of platelet counts with other bone-marrow derived parameters of inflammation including peripheral blood neutrophilia and lymphocytosis in an algorithm termed systemic immune inflammation index (SII) identifies a subset of early HCCs with higher circulating tumour cells at diagnosis and shorter survival following resection [51]. With the association between thrombocytosis and more aggressive clinical course of HCC being strengthened from independent studies [45], further mechanistic research is warranted to clarify whether such link is truly causative or rather represents an epiphenomenon in the course of HCC progression. The wide availability of antiplatelet agents in the clinic makes a stronger case for such stream of research to be prioritized.

15.2.3 Inflammation-Based Index (mGPS)

When acute tissue injury occurs, local recruitment and activation of cells pertaining to innate immunity including resident macrophages and neutrophils is facilitated by chemotactic mediators, which mainly act in both a paracrine and autocrine fashion to promote and sustain a local inflammatory response. Some of these mediators, including IL-6 and IL-1, have well-described endocrine effects, which include, for instance, thermoregulation (IL-1) and systemic modulation of complex biosynthetic processes [52]. An IL-6 peaks very early after acute tissue injury and, amongst other systemic effects, down-regulates albumin biosynthesis and induces the secretion of CRP [53]. CRP is a soluble acute phase reactant belonging to the pentraxin family and is a positive systemic regulator of the inflammatory response [52], modulating tumour microenvironment and promoting angiogenesis. Besides its recognized indirect role as a regulator of the tumour microenvironment, it is not completely understood whether CRP can directly impact on cancer progression. In a recent paper, a somatic mutation in the CRP locus was found to correlate with Wnt mutations in colorectal cancer cells, however the functional correlation and the significance of this is presently unknown [54]. On

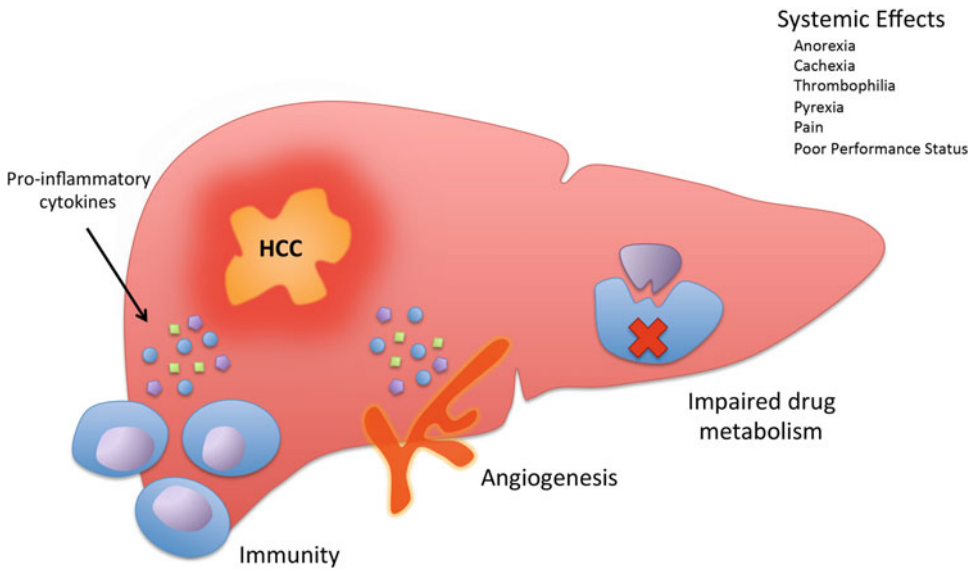

Fig. 15.1 The biologic relevance of the systemic inflammatory response in the prognosis and management of HCC

the other hand, other pentraxin family members like Pentraxin-3 (PTX3), have shown to facilitate tumour cell invasion in pancreatic cancer, suggesting novel mechanistic insights into inflammatory mediators as direct modulators of solid tumours progression [55] (Fig. 15.1).

The combination of hypoalbuminaemia and elevated CRP levels were utilized to derive the GPS, which has undergone subsequent modifications (mGPS) to improve its prognostic accuracy in patients with early as well as advanced stage cancers [24].

Interestingly, studies of large cohorts of patients have demonstrated mGPS to be a stage-independent prognostic predictor, associated with poor performance status (PS) [56].

While hypoalbuminaemia is notoriously determined at least in part by the underlying liver dysfunction that accompanies HCC, recent studies have shown that systemic inflammation plays an equally relevant role in influencing albumin levels [22], with anticancer therapies exerting a positive effect on albumin levels through modulation of the underlying inflammatory response [57]. The individual mechanistic role of CRP, the second component of the mGPS is not entirely understood. Given the redundancy of cytokine network signaling, it is not clear whether increased CRP is a bystander downstream effect of systemic cytokine excess or whether CRP secretion drives cancer progression. It is however clear that raised CRP levels are associated with poor prognosis of HCC both in the curative and palliative setting, and a recent study confirmed the inflammation based index (IBI) as a stage independent prognostic marker with superior accuracy to other inflammatory markers [22], with its dynamic changes following loco-regional therapies predicting for disease modulating effects and survival benefit in patients with intermediate stage HCC [57].

15.3 Inflammation-Based Prognostic Indices in the Curative Setting

According to the Barcelona clinic liver cancer (BCLC) algorithm, patients who present with unifocal asymptomatic HCC in the context of preserved liver function should be offered radical treatment in form of either percutaneous radiofrequency ablation (RFA) or hepatectomy [58]. Despite achievement of complete response, the predicted 5-year survival rates following resection for early stage HCC varies between 17 and 53 % [59]. Patients who have been radically treated for HCC have an overall lifetime risk of recurrence that approaches 70 % and it is felt that this percentage incorporates the risk emerging from primary progression of micrometastatic foci originating from the primary tumour as well as the establishment of new neoplastic clones stemming from the underlying cirrhosis [60].

As shown in Table 15.2, compelling evidence has demonstrated the NLR as an accurate predictor of OS and DFS following resection in patients with early stage HCC. In a large study including 958 patients, analysis of a subset of 150 resected tumour specimens revealed an elevated NLR to correlate with higher CD-163 positive peritumoral immune infiltrate, providing an insightful link between local and systemic inflammatory response [33]. Similar conclusions can be drawn for the NLR in the context of RFA, where deterioration of the score after treatment may anticipate early recurrence and subsequent mortality [61, 62]. Interestingly, most of the studies in early stage disease employed lower cut-off values in the NLR for prognostic stratification compared to advanced disease, raising the question as to whether the NLR can reflect a progressive, stage-dependent intensity and severity of the systemic inflammatory response and

Table 15.2 Summary table of the studies investigating the prognostic role of the neutrophil to lymphocyte ratio (NLR) and platelet to lymphocyte ratio (PLR) in patients with HCC

Study	Biomarker	Clinical setting	N	Comments
Gomez et al. (2008)	NLR	Resection	96	NLR $\geq$ 5 predicted worse DFS and OS
Halazun et al. (2009)	NLR	OLT	150	NLR $\geq$ 5 predicted worse DFS and OS
Bertuzzo et al. (2011)	NLR	OLT	219	NLR $\geq$ 5 predicted worse DFS and OS
Huang et al. (2011)	NLR NLR changes	TACE	145	NLR $\geq$ 3.3 pre-TACE predicted for worse OS. NLR increase 3 days post-TACE predicted for improved OS
Wang et al. (2011)	NLR	OLT	101	NLR $\geq$ 3 predicted for worse DFS and OS
Wang et al. (2011)	NLR	OLT	76	NLR $\geq$ 2.5 predicted for worse DFS and OS
Chen et al. (2012)	NLR NLR changes	RFA	158	NLR analysed as continuous explanatory variable predicted for worse OS but not DFS Elevated post-RFA NLR predicted for shortened DFS and OS
Pinato et al. [22]	NLR NLR changes	TACE	54	NLR > 5 at baseline predicted for worse OS Dynamic changes of NLR after TACE predict for OS advantage following TACE
Dan et al. [61]	NLR NLR changes	RFA	178	Post-RFA NLR worsening predicted for poorer OS and DFS
Fu et al. (2013)	NLR	Resection	282	NLR > 2 predicted for worse DFS and OS
Harimoto et al. (2013)	NLR	Recurrent HCC post OLT	167	NLR $\geq$ 4 predicted for worse OS
Limaye et al. (2013)	NLR	OLT	160	NLR $\geq$ 5 predicted for worse DFS and OS
Mano et al. [33]	NLR	Resection		NLR $\geq$ 2.81 predicted for worse OS and DFS and associated with higher peritumoral macrophage infiltrate
McNally et al. (2013)	NLR NLR changes	TACE	103	NLR > 5 at baseline predicted for worse OS Dynamic changes of NLR after TACE predict for OS advantage following TACE
Motomura et al. (2013)	NLR	OLT	158	NLR $\geq$ 4 predicted for DFS associated with higher IL-17 peritumoral expression
Oh et al. (2013)	NLR NLR changes	Mixed stages (37 % TNM I–II)	318	NLR > 2.3 predicted for worse OS and dynamic changes following treatment (mostly TACE) associated with radiologic tumor response
Xiao et al. (2013)	NLR	OLT	280	NLR $\geq$ 4 predicted for worse DFS and OS
Yoshizumi et al. (2013)	NLR	OLT	152	NLR > 4 predicted for worse DFS
Yoshizumi et al. (2013)	NLR	Salvage OLT in recurrent HCC after primary resection	104	NLR > 4 predicted for worse DFS after achieving complete response following salvage OLT
Zheng et al. [81]	NLR	Advanced HCC	65	NLR > 4 predicted for worse PFS and OS during treatment with Sorafenib
Liaso et al. (2014)	NLR	Resection	256	NLR > 2.31 predicted for worse DFS and OS
Shindoh et al. [69]	NLR	OLT	124	NLR > 2.4 predicted for worse DFS but with inferior accuracy (AUC 0.62) compared to AFP (0.88) and DCP (0.76)
Sullivan et al. (2014)	NLR	Mixed stages (15 % surgical candidates)	75	NLR measured as continuous variable did not associate with short term OS

Table 15.3 Summary table of the studies investigating the prognostic role of the GPS and IBI/mGPS in patients with HCC

Study	Biomarker	Clinical setting	N	Comments
Fujiwara et al. [63]	GPS	Resection	66	GPS associated with perioperative complications
Ishizuka et al. (2010)	hGPS[a]	Resection	300	hGPS associated with postoperative mortality
Ishizuka et al. (2011)	GPS	Resection	398	GPS predicted for worse OS
Kinoshita et al. [50]	NLR PLR GPS mGPS PI PNI	Mixed stages (55 % TNM I–II)	150	All inflammation-based indices emerged as univariate predictors of OS. GPS preserved independent prognostic power on MVA with greater accuracy established using AUC for predicting OS at 6, 12 and 24 months. The cut-off for NLR was ≥ 5 while for PLR was <150, ≥ 150 and ≥ 300
Morimoto et al. [80]	GPS	Advanced HCC	81	GPS predicted for OS in patients treated with sorafenib
Pinato et al. [22]	PNI	Mixed stages—mostly intermediate/advanced HCC	112 training set (BCLC-A 15 %) 68 validation set	PNI emerged as independent predictor of OS in both cohorts
Pinato et al. [22]	IBI NLR PLR	Mixed stages	112 training set (BCLC-A 15 %) 466 validation set (BCLC-A 56 %)	IBI emerged as most accurate predictor of OS Combination of IBI and CLIP resulted in improved prognostic accuracy
Horino et al. [64]	GPS	Resection	352	GPS predicted for perioperative complications and OS
Kinoshita et al. [20]	GPS	Mixed stages (prospective study)	150	GPS predicted for worse OS
Huang et al. (2014)	NLR GPS mGPS PI PNI	Resection	349	GPS emerged as most accurate predictor of OS. Combination of GPS and CLIP resulted in improved prognostic accuracy
Pan et al. (2014)	GPS	Resection	171	GPS predicted for worse OS and DFS
Pinato et al. (2014)	IBI IBI dynamic changes	TACE	64 training set 577 retrospective validation set[b] 76 prospective validation set	IBI and its dynamic changes following TACE predict for treatment-induced OS benefit The effect on patient's survival was validated prospectively

[a]hGPS was calculated using high sensitive CRP with a cutoff of 0.3 mg/dL
[b]In the Japanese sub-cohort IBI was calculated using high sensitive CRP with a cutoff of 0.3 mg/dL
CLIP Cancer of the Liver Italian Program score; *NLR* Neutrophil-to-lymphocyte ratio; *PLR* Platelet-to-lymphocyte ratio; *GPS* Glasgow prognostic score; *mGPS* Modified glasgow prognostic score; *IBI* Inflammation based index; *PI* Prognostic index; *PNI* Prognostic nutritional index; *BCLC* Barcelona Clinic Liver Cancer system; *TNM* Tumor node metastasis system; *TACE* Trans-arterial chemoembolization; *RFA* Radiofrequency ablation; *OLT* Orthotopic liver transplantation; *OS* Overall survival; *DFS*, Disease-free survival; *PFS* Progression-free survival; *MVA* Multivariate analysis of survival; *DCP* Des-gamma-carboxyprothrombin; *AUC* Area under curve

whether this should be accounted for by using different cut-off values across the diverse stages of HCC.

Similar figures have emerged from the study of albumin/CRP based prognostic algorithms in HCC, where derangement of these parameters prior to surgery predict for increased risk perioperative complications [63], longer operating times [64] as well as worse OS and DFS [65] (Table 15.3).

A number of studies have assessed the role of inflammation based indices in patients treated with orthotopic liver transplantation (OLT), a context where OS approaches 75 % at 4 years [66] and recurrence occurs in 8–15 % of all graft recipients fulfilling Milan criteria [67, 68]. Strikingly, the NLR emerged as a consistent and reproducible biomarker of shorter DFS and OS across a wide range of studies involving both Eastern and Western populations where the diverse selection criteria for OLT may exert a significant impact on survival outcomes. In one study by Shindoh et al., the accuracy of NLR employed with a cutoff of 2.4 emerged as independent predictor of DFS, albeit with inferior accuracy than alpha-fetoprotein (AFP) and des-gamma-carboxy-prothrombin (DCP) [69]. While promising and validating across different studies, the prognostic link between deranged inflammatory scores and survival in early stage disease mostly emerges from retrospective, single institution-based studies, which obviously limits clinical applicability in routine practice.

If validated in large, multi-institutional prospective studies, these findings may exert an impact on the management of HCC in the context of graft allocation and preoperative risk assessment of patients with resectable disease who, as a result of ongoing systemic inflammation, are at higher risk of perioperative complications and mortality.

Despite the plethora of studies investigating inflammation-based indices in early stage disease, only a minority has investigated the biologic background underlying a sustained systemic inflammatory response. Given the initial evidence suggesting that treatment-induced modulation of the cancer-related inflammatory response correlates with positive anti-tumour effects, dissecting the molecular foundations of such response may represent a source of targets to enable inflammation-based adjuvant treatment strategies in HCC.

15.4 Inflammation-Based Prognostic Indices in Advanced Disease

Patients with unresectable HCC have highly heterogeneous survival outcomes, ranging from 14 to 45 months in intermediate stage disease to [70], <3 months in BCLC-D [71]. Prognostication of intermediate stage HCC has been at the focus of intense research efforts, partly because of the inability of the BCLC algorithm to predict for survival benefit following trans-arterial chemo-embolization (TACE), the recommended treatment for patients with liver confined tumours and preserved liver function [72]. A number of prognostic algorithms have been proposed to guide the provision of TACE, none of which, however, have been validated or entered the clinical arena [73–75].

Interestingly, some of the proposed strategies rely on the measurement of CRP, which emerged as a strong prognostic determinant [17] and enabled the formulation of composite prognostic scores incorporating liver function and radiologic response to identify patients who should not undergo repeat TACE [76].

To further sustain the hypothesis that cancer related inflammation is a meaningful prognostic domain in HCC, a number of studies have shown that the dynamic changes in the NLR [77] and IBI [57] following TACE may reflect disease-modulating effects from treatment. Interestingly, normalization of inflammatory biomarkers post TACE reflect prolonged survival and better radiological response, therefore sustaining the hypothesis that suppression of a systemic inflammatory response may act as a surrogate biomarker for chemoembolization failure. With the exception of the IBI, whose prognostic role has been prospectively validated in diverse patient cohorts across Europe and Asia, other scores including the NLR have not undergone formal validation.

Following the observation that a single measurement of CRP at diagnosis predicts long term outcome in patients with HCC [17], a composite prognostic model incorporating baseline CRP as well as other variables to reflect radiologic response to TACE and progressive liver dysfunction has been proposed as a selection criterion (START strategy) to identify patients with intermediate stage HCC who are unsuitable for repeat TACE [76]. The ideal positioning of inflammation-based scores in the selection process of TACE candidates remains however unclear and deserves further evaluation in future studies, especially due to the emergence of recently qualified alternative prognostic models [73, 78, 79].

The prognostic qualification of inflammation-based indices in advanced disease has confirmed GPS and NLR as predictors of OS during treatment with Sorafenib [80, 81]. The studies, which included a relatively small number of patients, all collected retrospectively, have left the question of a comprehensive comparative analysis of all the utilized scores still unanswered, making it difficult to make recommendations for clinical use. In addition, the relationship between a pro-inflammatory status and toxicity from systemic treatment, a notion that has emerged from animal studies showing inflammation-driven repression of drug metabolism [82], remains unexplored in advanced HCC.

15.5 Inflammation as a Potential Therapeutic Target in HCC

On the basis of the association between systemic inflammation and HCC progression, pharmacologic manipulation of cancer-related inflammation is now hypothesized as a viable therapeutic strategy, with potential for integration with other systemic or loco-regional anticancer therapies.

15.5.1 Aspecific Modulation of the Systemic Inflammatory Response

Amongst the candidate anti-inflammatory strategies, only some have clearly identified targets or mechanisms of action to explain their antitumour effect, ranging from broad-spectrum approaches including corticosteroids, non-steroidal antiinflammatory drugs (NSAIDs), to more selective compounds that interact with specific molecular pathways.

Recent evidence suggests that treatment with dexamethasone, a widely used corticosteroid preparation prescribed as anti-inflammatory and to counteract cancer-related cachexia and anorexia in patients with advanced malignancies, may exert direct antitumour effects in animal models of HCC by inducing a metabolic switch from glycolysis to gluconeogenesis through regulation of 18β-hydroxysteroid dehydrogenase [83], a finding that warrants further clinical evaluation in prospective trials.

Aspirin has emerged as both a chemopreventative as well as a direct anticancer treatment as justified by both robust retrospective evidences [84]; the use of and a plethora of prospective randomized trials across a wide range of tumour types [85]. It remains unknown whether the anticancer properties of aspirin rely more on its antiplatelet effect, which in turn reduces T cell-mediated liver necro-inflammation [86], or rather on sustained cyclooxygenase (COX) inhibition, an increasingly relevant therapeutic target in HCC whose expression within the tumour microenvironment is harbinger of adverse prognosis [87]. Active repression of the NFκB-signaling pathway, which governs both tumour cell proliferation and inflammation and strongly relates to the pathogenesis and progression of HCC [88] is a putative target justifying the use of aspirin as well as other NSAIDs as disease-modulating agents [89].

15.5.2 Molecularly Targeted Modulation of Cancer-Related Inflammation

A number of targeted anti-inflammatory approaches have been under investigation in a wide range of metastatic malignancies including HCC and these include selective inhibition of specific pro-inflammatory pathways including IL-6, Tumour Necrosis Factor-α as well as chemokine receptors. These approaches have been comprehensively reviewed elsewhere [39], however none of these has translated into significant clinical improvement in the management of HCC.

Amongst the most promising therapeutic targets in HCC is the JAK/STAT pathway [90]. JAK is a well characterized intracellular kinase that is recruited to the active cytoplasmic domain of a number of growth factor tyrosine-kinase receptors and signals downstream via STAT protein dimerization and nuclear migration [91]. Selective inhibition of JAK is now clinically achievable and a recent trial of ruxolitinib, an oral JAK-1 and 2 inhibitor administered in combination with capecitabine has demonstrated a significant PFS and OS advantage in a subset of pre-treated pancreatic cancer patients with evidence of ongoing systemic inflammatory response as measured by the mGPS at study baseline. The preliminary results of this trial suggest that inflammation-based stratified therapies yield the potential to optimize drug development and clinical outcomes in patients with advanced malignancies [92].

Targeting TGF-β related signaling is regarded as another encouraging focus of therapeutic development in HCC due to the potential of this pathway to modulate both tumour progression and the surrounding microenvironment by altering neoangiogenesis as well as restoring the immune cell dysfunction that accompanies the molecular progression of HCC [32]. A number of selective inhibitors of TGF-β signaling are in clinical development and act on the pathway either by ligand deprivation (monoclonal antibodies) or by selective inhibition of the TGF-β receptor intracellular kinase domain [93]. Metelimumab and lerdelimumab are recombinant human IgG_4 antibodies that respectively bind to TGF-β1 and 2 isoforms, while amongst the intracellular kinase inhibitors, the TGF-βRI inhibitor galunisertib (LY2157299) has emerged as a lead compound following completion of first time in man evaluation [94], having been now prioritized for proof-of-concept studies across a wide range of solid tumours including HCC.

15.5.3 Immunotherapy

A significant consequence of systemic inflammation is represented by the progressive suppression of anti-tumour specific immunity. The recent advances of immunotherapy in advanced-stage melanoma have extended the clinical evaluation of immune checkpoint inhibitors to HCC. The rationale behind the use of immune-modulating agents in HCC, including anti-cytotoxic T lymphocyte associated antigen 4 (CTLA-4) antibodies like ipilimumab or PD-1/PD-L1 antagonists such as nivolumab or pembrolizumab, stems from the observation that HCC originate from a background of chronic inflammation, rich in tumour-associated antigens. It is therefore hoped that modulation of the adaptive T cell response against the HCC neo-epitome may improve clinical outcomes of patients with advanced disease [95]. The clinical development of immune checkpoint inhibitors is still at its earliest phases in HCC, with one initial phase I study of Tremelimumab, an anti-CTLA-4 antibody; producing disease control in 76 % of

a small group of 20 patients with hepatitis C related HCC [96]. With more immune-modulating compounds enriching the pipeline of HCC drug development [95], a number of challenges will accompany the full development of immune checkpoint inhibitors within the specific context of HCC. Unlike melanoma, the concurrent presence of chronic hepatotropic viral infections may pose a potential risk of hepatitis flares, which could potentially worsen pre-existing liver dysfunction. Secondly, the emergence of potentially life-threatening immune-mediated complications from checkpoint inhibitors might discourage their use in specific subsets of patients with autoimmune comorbidities [97]. Thirdly, patient selection based on molecular prediction of response is an anticipated need for PD-L1 directed therapies, where greater benefit seems to be anticipated in PD-L1 expressing tumours, a point which might reshape the clinical need for a tissue-based diagnosis, a largely abandoned practice in advanced disease due to the increased accuracy of radiologic criteria.

15.6 Conclusion

While the interplay between local, systemic inflammation and the progression of HCC is now a consolidated concept in determining the pathogenesis and prognosis of the disease, a number of key questions still remain unanswered.

From a clinical standpoint, the use of inflammation-based biomarkers, although inexpensive and universally available, competes with other prognostic algorithms. In conjunction with the lack of prospective validation that applies to most of the studied indices, the routine clinical use of inflammatory biomarkers is hindered by their perceived limited potential to inspire clear changes in the management of patients with HCC. A strong indication that inflammation-based indices may yield practice-changing information comes from the results of the RECAP trial (NCT01423604) [92], where patient stratification by pre-treatment mGPS was able to detect treatment-induced changes in patients' survival not otherwise captured when analysing the whole intention-to-treat population, paving the way for further phase III studies in advanced pancreatic cancer (Janus 1 trial NCT02117479).

In the specific context of HCC, a patient subpopulation where inflammation-based indices might be most useful is the intermediate or BCLC-B stage, where survival outcomes are highly variable as a result of a varying grade of tumour burden and liver dysfunction [98]. The identification of patients who are less likely to benefit from locoregional treatments has emerged as a clinical priority; hence an accurate, prospective comparison of inflammatory markers with other available clinical scores is warranted [99].

Advanced HCC is a further area of clinical development of inflammatory scores. Firstly, documented evidence of deranged inflammatory indices at initiation of planned treatment or their worsening over time may provide an objective measure to rationalize the provision of Sorafenib to patients depending on its predicted efficacy, an important aim in advanced HCC where pharmaco-economic implications have been a pressurizing issue across several healthcare systems.

In addition, a number of published reports now emphasize the relationship between systemic inflammation and toxicity from systemic anticancer treatments, which stems from modifications in pharmacokinetic parameters including volume of distribution direct hepatic repression of cytochrome P450 metabolism, a major detoxification pathway involved in Sorafenib clearance [82, 100]. Whether or not an inflammatory diathesis may prelude excessive toxicities from Sorafenib it remains to be ascertained.

While research addressing the role of systemic inflammation is expanding, an improved understanding of its role in the natural history of HCC is expected to aid clinicians and scientists to deconstruct the molecular portrait of HCC, with positive implications in the routine prognostic assessment, management planning as well as in drug development, facilitating the provision of personalised medicine in the context of early as well as more advanced stage HCC.

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Hepatocellular Carcinoma Associated with Hepatitis B Virus

16

Inbal Houri and Oren Shibolet

Contents

16.1 Introduction

Hepatocellular carcinoma (HCC) is ranked as the fifth most common malignant neoplasm in the world [1], and the third most common cause of cancer death worldwide [2]. HCC's global incidence is approximately 600,000 new cases annually, almost 85 % of these in developing countries. In fact, it is the third most commonly diagnosed cancer among males in developing countries and the second leading cause of death among that population. The vast majority of deaths from HCC occur in East Asia, and 50 % are estimated to occur in China alone. 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 [3, 4]. Although less common in developed countries, it is still a major cause of morbidity and mortality. Globally, about 80 % of HCC is considered to be causally associated with chronic infection with HBV [5, 6].

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16.2 Hepatitis B Virus

16.2.1 Background

HBV is a double-stranded DNA virus belonging to the Hepadnaviridae (hepatotropic DNA virus) family, and is classified as hepadnavirus type 1. The intact virus consists of an outer coat component of hepatitis B surface antigen (HBsAg) and an inner core component of hepatitis B core antigen (HBcAg) [7–9]. Hepatitis B e antigen (HBeAg), that is also a product of the C gene, circulates in the blood during periods of high replication [10, 11].

The hepatitis B viral genome is approximately 3200 base pairs in length, is partially double-stranded, and uses a retroviral mode of replication [12, 13]. The viral genome contains genes that code for HBsAg, HBcAg, and DNA polymerase [9, 14]. An additional X gene codes for hepatitis B x antigen (HBx), a protein that is capable of transactivating the transcription of both viral and host genes [15, 16].

HBV predominantly infects hepatocytes, but reservoirs of the virus were found in extra hepatic sites including lymph nodes, bone marrow and circulating lymphocytes, explaining recurrence after liver transplantation [17, 18]. Infection of hepatocytes is by specific binding of the envelope viral protein (specifically the preS1 domain) to a bile salt transporter sodium taurocholate co-transporting polypeptide (NTCP) [19].

Eight genotypes of HBV have been identified (A–H), classified by the subtype-specific antigens on the HBsAg, and their distribution varies geographically. Genotype A is more prevalent in Europe, North America, and Africa, while genotypes B and C are dominant in China and East Asia, where vertical transmission is more common [20, 21]. Genotype D is found most commonly in Europe and

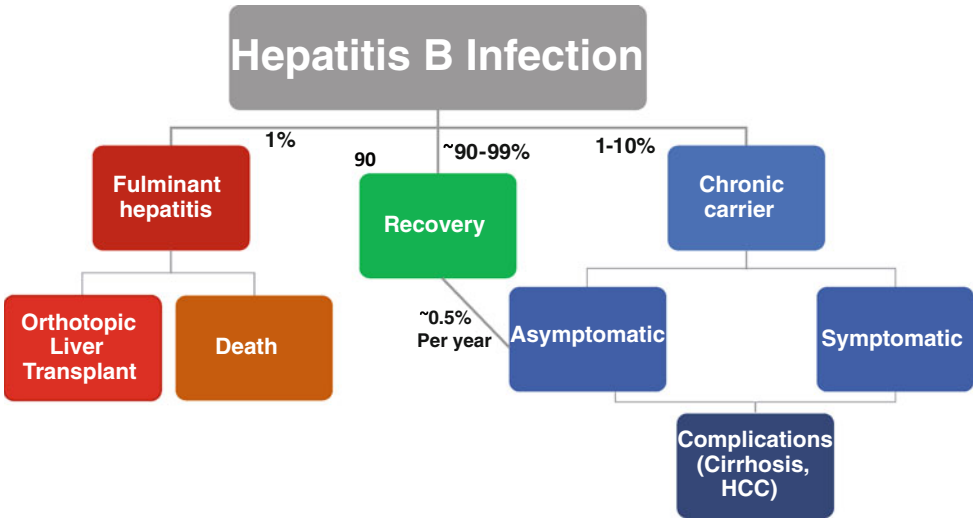

Fig. 16.1 Natural history of HBV infection

Mediterranean countries, genotype E predominates in West Africa, and Genotype F in Central and South America. The specific HBV genotype is associated with clinical characteristics such as disease progression and response to interferon therapy. Genotype B, for instance, appears to be associated with a less rapidly progressive liver disease and a lower likelihood or delayed appearance of HCC [22–25], as opposed to genotype C [26], while patients with genotype A are more likely to respond to interferon therapy [27, 28].

HBV is carried in blood as well as other body fluids. The main routes of transmission are sexual intercourse, perinatal transmission, and parenteral exposure. Perinatal transmission occurs from chronically infected mothers or during acute infection at the third trimester or early postpartum, and is more common in developing countries. The precise mode of perinatal transmission is unknown but most probably occurs at the time of delivery. Risk for infection correlates with viral activity, as 85 % of HBsAg-positive mothers who are HBeAg-positive will transmit the virus to their offspring, whereas mothers who are positive for anti-HBe do so much less frequently (31 %) [29]. Additionally, maternal HBsAg titers correlate with the risk for transmission [30].

The natural history of hepatitis B infection differs by the age of acquisition of the infection. Nearly 90 % of exposed newborns will become chronic carriers, compared to 50 % during infancy and 20 % during early childhood [11, 31–33]. Among healthy adults exposed to HBV infection, 90–99 % have a full recovery, 0.1–1 % develop acute fulminant hepatitis and 1–10 % become chronic carriers (Fig. 16.1). In chronic carriers, the rate of spontaneous HBsAg clearance is approximately 0.5 % per year [34–36].

The natural evolvement of chronic infection can be divided into four phases—immune tolerant phase, characterized by HBeAg (+), high levels of HBV DNA, normal serum aminotransferases and minimal or no inflammation on liver biopsy; immune active/clearance phase, which manifests with elevated serum aminotransferases and active inflammation on liver biopsy; low-replicative phase (inactive carrier), with seroconversion from HBeAg (−) to anti-HBe (+), low serum HBV DNA and normal aminotransferase levels, and finally the HBeAg (−) hepatitis phase (HBV reactivation) which presents high serum HBV DNA and active inflammation [33, 37]. Seroconversion from HBeAg to anti-HBe rates differ by patient age and are approximately 10 % per year for adults but <5 % for patients with perinatally acquired infection [33, 34].

Although chronic HBV carriers have been infected for extended periods of time, most do not have symptoms. Many patients are found to have chronic hepatitis B incidentally during 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 liver functions [38].

15–30 % of chronic hepatitis B patients 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.

16.2.2 HBV Epidemiology

Hepatitis B is a common infection worldwide, with approximately one third of the world population having serological evidence of past/present infection. At least 350–400 million are chronic HBV carriers worldwide [4] and globally, there are around 4.5 million new infections per year [39].

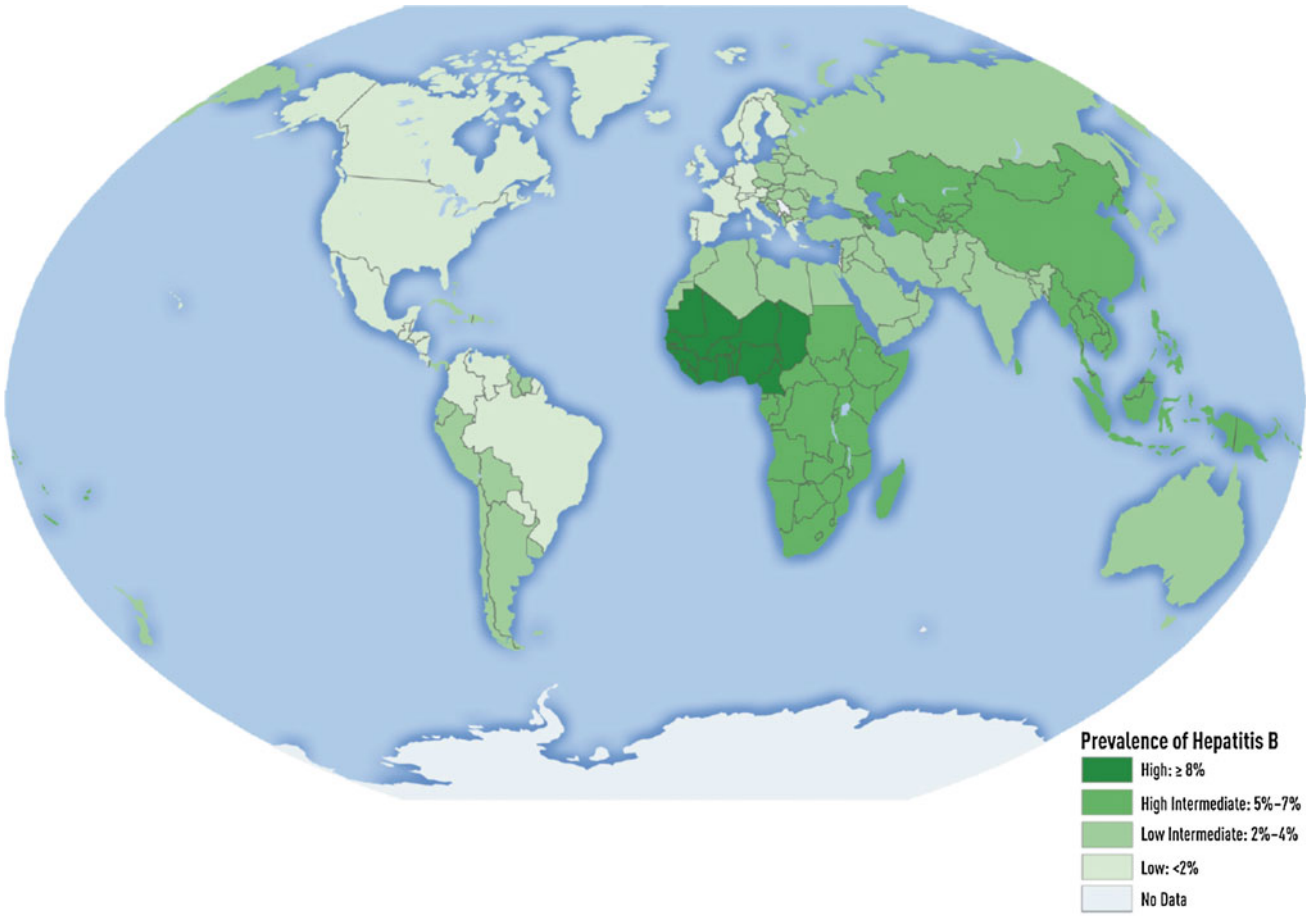

Fig. 16.2 Prevalence of chronic hepatitis B virus infection among adults

The prevalence of hepatitis B differs by geographical region, and countries are divided into high (>8 % prevalence), intermediate (2–7 %) and low (<2 %) endemicity for the virus (Fig. 16.2). Areas considered highly endemic include central and southeast Asia and sub-Saharan Africa. Regions of intermediate 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 [40, 41].

The skewed distribution of HBV infection is most likely due to the different modes of transmission. In the hyperendemic 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 parenteral exposure are the major modes of transmission [31].

16.2.3 Treatments for HBV

Management of chronic hepatitis B is aimed at HBsAg loss and decrease of active virus replication. Treatment for HBV has come a long way in the past decades, changing the prognosis of chronic hepatitis B carriers. Currently, there are seven FDA-approved antiviral therapies, six of them used routinely (interferon treatment was replaced by pegylated interferon) (see Table 16.1).

Interferon-α was the first approved treatment for chronic hepatitis B. In IFN responders, a hepatitis-like flare, presumably due to immune activation, accompanies HBeAg seroconversion. Nowadays, pegylated-IFN is used in clinical practice. This drug is given by injection weekly, and is more effective as well as more convenient for patients compared to regular IFN. Despite its proven efficacy it is relatively poorly tolerated because of its side effect profile and mode of administration. HBeAg seroconversion after 1 year is achieved in ~30 % of patients.

Table 16.1 Antiviral agents currently in use for HBV therapy in the U.S.

Drug	FDA approval
Interferon (IFN)	
(regular) IFN-α	1992
Pegylated interferon	2005
Nucleoside analogues (*NUCs*)	
Lamivudine	1998
Adefovir	2002
Entecavir	2005
Telbivudine	2006
Tenofovir	2008

Nucleoside analogues (NUC) are oral medications that suppress viral replication by inhibiting reverse-transcriptase activity. They are well tolerated and effective in viral suppression. HBeAg seroconversion after 1 year is achieved in ~20 %, 30 % after 2 years, and 50 % after 5 years. One of the major problems of NUCs is development of resistance, which can be combatted by adding on or switching to a different NUC.

16.3 HBV and HCC

Half of the world's population lives in the regions of high incidence for HCC, which also coincide with endemic regions for HBV infection. Worldwide, HBV accounts for 54 % of HCC cases, and in Asia and Africa it accounts for 70 % of cases [42].

A large number of epidemiologic studies [43–47] documented the causal association of HCC with HBV. In 1981, the landmark study by Beasley et al. [48] lucidly demonstrated the relationship between HBV and HCC.

During the past decades, the incidence of HCC has decreased in some areas in East Asia such as in Taiwan, Korea and Thailand, as well as other highly endemic areas such as Alaska [49–53]. It is believed that effective HBV vaccination programs have contributed to the reduction (see below). However, the opposite phenomenon was reported in some countries in Europe, North America and Oceania [6, 54]. This increasing incidence of HCC is attributed to HCV and non-alcoholic fatty liver disease (NAFLD)-associated cirrhosis and also the immigration of HBV carriers from endemic regions.

Increasing incidence of HCC in the United States is clearly illustrated by the HCC incidence in California, the state that has the largest Asian American population. As shown in Table 16.2, 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 them [55]. Although Asian Americans constitute only 4.5 % of the total USA population, they constitute nearly half of the total HBV chronic carriers in the U.S.A [55–57].

Asian immigrants in the U.S.A show high HBV carrier rates reminiscent of their homelands, and accordingly have high risk for sequelae of chronic hepatitis B infection, such as cirrhosis and HCC. In fact, the HBV carrier rate among first generation immigrants is similar to that of people living in their native lands [38, 58, 59].

Currently, the estimated prevalence rate of chronic HBV carriers ranges between 5–13 % among Asian Americans. In contrast, HBV carrier rate for the general U.S. population is less than 0.3 % [60].

Tong and Hwang conducted a prospective study of 207 HBsAg (+) Asian American patients with chronic hepatitis [55]. 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) [3] and those by Liaw et al. [61] (826/100,000 for all ages and 2768/100,000 for patients older than 35 years of age). Nonetheless, it is important to point out that Beasley followed asymptomatic carriers, and Tong et al. and Liaw et al. followed patients with active hepatitis. Most HCC occur in patients that are between 40 and 60 years of age [5] although there are some exceptions including childhood HCC described in Taiwan [62] and in native Americans in Alaska [53].

16.3.1 Pathogenesis of HCC in HBV

HCC is strongly associated with chronic liver disease, and is uncommon in the absence of inflammation and fibrosis [63]. Hepatic carcinogenesis is a long term, multistage disease process encompassing multiple genetic alterations, including activation of cellular oncogenes and inactivation of tumor suppressor genes [64, 65].

16.3.1.1 Risk Factors for the development of HCC in Chronic HBV carriers

HBV infection carries an increased risk of developing HCC. Several risk factors confer higher risk in HBV carriers (Table 16.3).

Although HBV-associated HCC does not always progress through cirrhosis [66], patients with cirrhosis are at the highest risk for developing HCC. Earlier studies in Japan found that prevalence of overt cirrhosis among patients with HBV-related HCC was 50–60 % [67, 68], but a later study by Yang et al. [69] found that most patients with HBV-related HCC have evidence of cirrhosis (73.4 % when using stringent clinical criteria and 93.8 % using the most inclusive criteria). Interestingly, all Caucasian patients had cirrhosis, compared

Table 16.2 Five most common cancers in males by race/ethnicity, California, 2007–2011

	Rank				
	1	2	3	4	5
Asians					
Laotians	**Liver**	Lung	Colorectal	Prostate	Stomach
Vietnamese	Lung	**Liver**	Prostate	Colorectal	Lymphoma
Chinese	Prostate	Lung	Colorectal	**Liver**	Lymphoma
Korean	Prostate	Colorectal	Lung	Stomach	**Liver**
Filipino	Prostate	Lung	Colorectal	Lymphoma	**Liver**
Non-Asians					
White	Prostate	Lung	Colorectal	Melanoma	Bladder
Hispanic	Prostate	Colorectal	Lung	Lymphoma	Kidney
Black	Prostate	Lung	Colorectal	Kidney	Bladder
American-Indian/Alaska native	Prostate	Lung	Colorectal	**Liver**	Kidney

California Cancer Facts and Figures 2014, American Cancer Society [222]
Bold represents liver cancer

Table 16.3 Risk factors of HCC among HBV carriers

Host-related
Cirrhosis
Age >40 years
High-endemicity areas
Male sex
Serum ferritin >300 ng/ml
Alcoholism
Family history of HBV infection
Viral-related
Genotype
↑ Serum HBV DNA

to a smaller proportion of Asian patients. A systematic review by Fattovich et al. [70] estimated the rates of HCC in East-Asian countries among inactive carriers (HBsAg (+), anti-HBe (+), normal ALT) to be 0.5 per 100 person years, 0.6 per person years in patients with chronic hepatitis but without cirrhosis, and 3.7 per 100 person years in patients with compensated cirrhosis. Importantly, rates were lower in developed countries—0.02 per 100 person years in inactive carriers, 0.3 per person years in patients with chronic hepatitis but without cirrhosis, and 2.2 per 100 person years in patients with compensated cirrhosis.

Earlier age at infection with HBV is associated with higher risk for HCC [71], in correlation with evidence suggesting that it takes 20–40 years to develop HCC from the time of infection [48]. Patients from areas of high HBV endemicity are at higher risk for HCC, presumably because they were likely infected early in life and thus had a longer duration of chronic infection or cirrhosis [63, 70].

Family history of HBV infection has also been found to be a unique risk factor for early onset of HCC [72]. Males have a higher incidence rate for HCC among HBV carriers with a 4:1 male to female ratio [2, 73]. The biologic basis for the gender difference in the risk for HCC is not well understood; however, male hormones [74], differences in body iron storage [75], and additional risks, such as alcohol consumption [76, 77] and smoking [77, 78], have been considered to be contributory factors. Older age is also considered a risk factor, since HCC occurs most commonly later in life [5].

Iron has long been considered a factor contributing to hepatic damage and inflammation via generation of reactive oxygen species (ROS). A sustained serum ferritin level greater than 300 ng/ml was shown to confer a higher risk for HCC. In a longitudinal follow-up study of 249 Korean patients with chronic hepatitis B and cirrhosis, Hann et al. [75] observed that chronic hepatitis B infected males with sustained serum ferritin >300 ng/ml had a 50 % chance of developing HCC compared with 20 % risk for HCC for those with lower serum ferritin levels. Further studies by the same group clearly demonstrated the tumor enhancing effects of iron [79–82].

Multiple studies have shown that increased viral activity [83] and a persistently high level of viral DNA is a strong predictor for HCC development [84–86]. Chen et al. conducted a large-scale longitudinal study of 3653 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 the 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 $\geq$1,000,000 copies/ml.

16.3.1.2 Molecular Biology of HCC associated with HBV

Recent advances in molecular techniques have markedly improved our understanding of HBV-associated hepatic carcinogenesis. The effects of HBV on HCC development can be divided to direct oncogenic mechanisms and indirect effects, via chronic liver inflammation and cirrhosis [87, 88].

Many of the pathogenic mechanisms of the virus are mediated by hepatitis B x protein (HBx), which is a small 154 amino acid protein with transcriptional regulatory activity [89]. The important role of HBx in HBV infectivity was realized when woodchucks injected with HBx-deficient viruses did not develop infection [90, 91]. Furthermore, HBx expression is abundant in the livers of patients with chronic liver disease [92, 93], and expression levels correlate with progression of liver inflammation and cirrhosis [94]. There is accumulating evidence that HBx is important in supporting virus replication and in the pathogenesis of chronic inflammation and HCC [95]. HBx augments viral replication and thus, by maintaining high viral DNA levels it contributes to hepatic carcinogenesis [96].

Hepatitis B and Fibrosis

A salient aspect of chronic liver disease is the development of fibrosis. There is increasing evidence that HBx expressing hepatocytes contribute to pro-fibrotic signaling. For example, HBx has been shown to up-regulate the expression of transforming growth factor beta 1 (TGF-β1) in HBx transgenic mice [97] and in liver cell cultures stably transfected with HBx [98]. In the normal liver, TGF-β1 signals through a group of proteins known as Smads, which inhibit hepatocellular growth and maintain homeostasis [99]. TGF-β1 has long been implicated in promoting fibrosis via activation of hepatic stellate cells, transforming them into myofibroblasts [100]. Additionally, in the presence of HBx, Smad protein transcriptional activity was enhanced, especially in activation of genes involved in extracellular matrix (ECM) production [101]. Besides altering Smad signaling directly, HBx activates other signaling molecules, such as NF-κβ, PI3K, AP-1, and ras/raf/MAPK, among others [102, 103] that override negative growth regulation. Importantly, up-regulated expression of TFG-β1 stimulates expression of platelet derived growth factor (PDGF), constitutively activating β-catenin, which may act as an oncoprotein [104].

Immune Response to HBV Infection and Necro-inflammation

HBV is a non-cytopathic virus [105]. Damage to infected hepatocytes is in large part immune-mediated, mostly via CD8+ cytotoxic T cells [106]. HBV clearance is regulated by the adaptive immune system, with CD4+ and CD8+ T cells mediating immune clearance and B cells supplying protective immunity by generation of neutralizing antibodies [107, 108]. In those patients with chronic infection, immune response is inadequate for viral clearance but still causes liver injury [109]. The factors governing immune clearance versus immune tolerance are not fully understood. One suspected mechanism in chronic infection is faulty modulation of regulatory T cells, which causes down regulation of T cell cytotoxicity [110–112]. Additionally, HBV disrupts toll-like receptor (TLR) signaling, disrupting the response of the innate immune system [113]. Another suggested mechanism of immune-resistance is by up-regulation of URG7 (up-regulated gene, clone 7) via HBx *trans*-activation, which was shown to confer resistance to Fas and tumor necrosis factor alpha (TNFα) mediated apoptosis [114, 115]. Further analysis showed that URG7 blocked apoptotic signals at the level of caspase 8, which is shared by Fas and TNFα signaling pathways.

In the presence of persistent infection and a chronic but ineffective immune response, the liver's unique regeneration ability causes repeated compensatory proliferation, eventually leading to cirrhosis and HCC [116].

Direct Oncogenic Effects of HBV and HBV Genome Integration

As mentioned above, patients with chronic hepatitis B are at risk for HCC even in the absence of cirrhosis [66], thus presenting the direct tumorogenic effects of the virus [117].

HBx was shown to localize to mitochondria [118], where it triggers oxidative stress [119] and production of ROS. Generation of ROS causes endoplasmic reticulum stress, which in turn compromises protein-folding ability leading to apoptosis and liver damage.

There is evidence that HBx 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 [120–124].

Another direct oncogenic mechanism of HBV affects regulation of cell growth. During chronic infection, fragments of HBV DNA integrate into the human genome at multiple sites [125, 126]. HBV DNA integration can also occur in occult infection [127]. Most of these integrated fragments span the HBx gene of HBV [128, 129].

HCC was shown to harbor mutations that may affect multiple cellular processes. These include inactivating tumor suppressor pathway components, activating oncogenes, and/or blocking DNA repair.

For example, p53 is a tumor suppressor gene that is activated during cellular stress to allow cell cycle arrest and initiation of DNA repair mechanisms [130]. It was shown that HBx binds to and functionally inactivates p53 [131–133], thus blocking p53 dependent transcription coupled repair [132] and

inhibiting nucleotide excision repair [134]. Disruption of cellular repair mechanisms leads to the accumulation of mutations, with those commonly found in HCC including inactivating point mutations of p53 [135, 136] and activating point mutations in β-catenin [137] which then acts as an oncogene in HCC. HBx is also implicated in constitutively activating other genes that appear to contribute to multi-step hepatocarcinogenesis, such as those encoding cyclin D1 [138, 139], URG4 [140] and URG11 [141]. Additionally, HBx down-regulates transcription of $p21^{WAF1/SDI1/CIP1}$ [142], a senescence factor that also inhibits cell cycle progression. Finally, HBx has been shown to overcome RAS oncogene induced senescence [143]. An emerging mechanism in HBV pathogenesis, including carcinogenesis, is by microRNAs (miRNA) [144]. HBV-related HCC cells have decreased expression of miRNAs that are known to regulate genes related to cell death, and increased expression of miRNAs that down-regulate inflammation [145].

HBx also has epigenetic effects on gene expression in liver cells. HBx activates expression of DNA methyltransferase 1 (DNMT1), in addition to other DNMTs, resulting in altered DNA methylation patterns in the chronically infected liver and in HCC [146, 147]. In this context, HBx activation of DNMT1 has been shown to promote hypermethylation of the promoter encoding E-cadherin, effectively suppressing E-cadherin expression [148, 149]. 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.

In a study by Boyault et al. [64] HCC's were classified to 6 groups (G1-6) according to transcriptome analysis. HBV-related HCC's were molecularly distinct from other HCC's, and were classified in groups G1-2. Clinically, G1 tumors were characterized by low HBV DNA levels, high serum α-fetoprotein (AFP), younger age and African origin. Frequent AXIN1 mutations were also seen. These tumors had genes expressed during development. G2 tumors were related to HBV infection with high HBV DNA levels, and frequent local and vascular invasion. Additionally there were frequent mutations in p53. Both G1 and G2 tumors had AKT pathway activation, in G1 via over expression of insulin growth factor 2 (IGF2) and in G2 via PIK3CA mutations.

A later study by Amaddeo et al. [150] further characterized genomics of HBV-related HCC's. A high frequency of p53 mutations was found compared to HCC's of other etiologies. Interestingly, more than 70 % of tumors harbored inactivation mutations in HBx gene, in contrast to non-tumor liver tissues. Among HBV-infected patients with additional risk factors, molecular characteristics were different, suggesting an alteration in carcinogenic mechanisms.

16.4 Prevention of HCC Related to HBV

As hepatitis B infection accounts for more than 50 % of all HCC cases worldwide [42], targeting HBV is an effective way to reduce global HCC burden. The targeted approach to prevent HBV-related HCC is aimed at 3 populations. Primary prevention in uninfected individuals, secondary prevention in chronic hepatitis B infected individuals, and tertiary prevention for HBV carriers who have already developed HCC [151].

16.4.1 Primary Prevention of HCC

Primary prevention of HCC aims to prevent HBV infection altogether among uninfected individuals, thereby reducing the risk for HCC development. This is accomplished by universal vaccination.

The first active vaccine was introduced in the 1980s [152], and was initially offered only to high-risk populations [153]. In 1991 the World Health Organization (WHO) recommended universal vaccinations in all countries [154, 155]. As of 2013, HBV vaccination is part of the vaccination schedule in 183 countries.

The impact of the universal vaccination plans on HCC development was significant [156, 157]. Initially, reduction of prevalence rates was seen in endemic countries, and later the effects on long-term morbidity and mortality were also documented. In Taiwan, where a universal vaccination plan was implemented in 1984, the prevalence of HBsAg among persons younger than 15 years decreased from 9.8 to 0.7 % after 15 years [158]. In Gambia, a study comparing HBV carrier status between vaccinated and unvaccinated 9-year-old children showed prevalence of 0.6 and 10 %, respectively [159]. Studies in China and Korea had similar findings [49, 160]. The benefits of universal vaccination programs were proven also in low or intermediate endemicity regions such as Italy [161].

In accordance with the decrease in chronic HBV carrier status, there has been a decrease in HCC prevalence following implementation of universal vaccination. Studies in Taiwan showed a decrease in incidence of HCC among children born after the implementation of the vaccination program compared to those born before, from 0.7 per 100,000 children to 0.36 [50, 51]. Similar findings were shown in studies in Korea, Thailand and Alaska [49, 52, 53], and in the latter elimination of HCC and acute Hepatitis B were achieved [162].

For unvaccinated patients who are exposed to hepatitis B, post-exposure prophylaxis is implemented using HBIG and the standard active vaccine.

16.4.2 Secondary Prevention of HCC

Secondary prevention of HCC is aimed to prevent HCC in the 400 million patients who are chronic HBV carriers. This is accomplished by effective antiviral treatment to reduce viral replication, thereby reducing the risk for HCC, and by surveillance programs to detect tumors at early stages when curative treatments are optional.

The first important step is to identify all HBV carriers. Current recommendations for groups who should be screened are summarized in Table 16.4 [163, 164].

16.4.2.1 Antiviral therapy

As mentioned above, one of the major risk factors for HCC in HBV carriers is a persistently high viral load. This suggests that treatment aimed to reduce viral load may decrease the risk for long-term sequelae, including HCC.

Current treatments to reduce viral replication include pegylated interferon, nucleotide (zide) (NUC) analogues and combination therapies. Studies that assessed the effect of IFN treatment on prevention of HCC yielded mixed results. Some showed significant reduction of HCC risk with IFN treatment [165–168] while others showed minimal or no effect [169–172]. One long-term, randomized controlled study reported treatment with natural lymphoblastoid interferon-alpha (IFN-αnl), recombinant IFN-α2a and placebo [173]. HCC was detected in 1.5 % of the IFN-αnl group, 3.7 % of the IFN-α2a group and 14.7 % of the control group ($p < 0.05$). In another long-term study that followed 411 chronic hepatitis B patients, of whom 208 were treated with IFN-α and 203 were controls, 4.3 and 1.0 % of patients in the IFN group and controls, respectively, developed complications of cirrhosis and HCC, but without statistically significant differences between the groups ($p = 0.062$) [169]. To overcome the variability in response seen in multiple small studies, a number of meta-analyses were performed. One such meta-analysis in 2001 did not show an effect for IFN in preventing HCC in several European studies [174]. In contrast, a few newer meta-analyses found significant risk reductions with IFN treatment [175–177]. Although some controversy remains, the overall conclusion is in favor of IFN treatment for viral suppression and reduction in HCC risk, in accordance with current treatment guidelines.

Table 16.4 Populations recommended for HBV screening

High and intermediate endemicity regions
All people
Low endemicity
Immigrants or adopted children from intermediate or high-endemicity regions
Household and sexual contacts of HBsAg-positive persons
Persons who have ever injected drugs
Persons with multiple sexual partners or history of sexually transmitted disease
Men who have sex with men
Inmates of correctional facilities
Individuals with chronically elevated ALT or AST
Individuals infected with HCV or HIV
Patients undergoing renal dialysis
All pregnant women
Persons needing immunosuppressive therapy

The majority of studies on NUC treatment have shown beneficial effects in prevention of HCC [178]. Prospective and retrospective studies of large numbers of chronic HBV patients with advanced liver disease have demonstrated that treatment with lamivudine (LAM) both delays disease progression and reduces HCC incidence. In a randomized controlled trial (RCT) by Liaw et al., 651 chronic hepatitis B patients with advanced fibrosis and cirrhosis were randomized to receive antiviral agents, LAM or placebo (2:1). Within 3 years, treatment with LAM not only delayed disease progression but also reduced the incidence of HCC [179].

Case-control studies demonstrated similar beneficial effects. Matsumoto et al. [180] in a retrospective study of 2795 individuals with chronic hepatitis B assessed the effectiveness of LAM in preventing HCC. 657 patients received LAM and the remaining 2138 served as controls. The mean follow-up period was 2.7 years for the LAM group and 5.3 years for the controls. Annual incidence of HCC in the LAM group was 0.4 %/patient/year compared to 2.5 %/patient/year in controls ($p < 0.001$). Yuen et al. [181] compared a group of HBeAg (+) individuals without cirrhosis treated with LAM to controls, with significantly lower rates of HCC and cirrhosis among the LAM-treated participants. In another study by Eun et al. [182] 872 chronic hepatitis B patients treated with LAM were compared to 699 historical controls that were not treated. The annual incidence of HCC was 0.95, 2.18, 5.26, and 4.10 % in patients with sustained viral suppression, viral breakthrough, suboptimal response, and the control group, respectively. A retrospective study from Greece compared 201 LAM-treated patients, of whom 79 of 109 without virological remission received adefovir as rescue therapy, with 209 patients treated with IFN-α and 195 untreated patients [183]. The liver-related survival in LAM-treated patients was significantly better compared with untreated patients or non-sustained responders to IFN-α, and similar compared with IFN-α sustained responders. Beneficial effect of LAM treatment was found in additional studies in Italy [184] and a recent study from Japan [185].

Newer NUCs show even more promising results. A large case-control study from Japan compared the incidence of HCC in entecavir (ETV)-treated patients, LAM-treated patients (with no rescue therapy) and non-treated historical

controls. The cumulative HCC incidence rates in cirrhotic patients at 5 years were 7, 22.2 and 38.9 %, respectively [186]. Additional studies comparing ETV-treated patients to untreated controls showed similar results [187, 188].

In a meta-analysis by Sung et al. [175] five studies comparing NUCs to placebo were analyzed. The use of NUCs (mostly with LAM, with some patients receiving adefovir rescue) reduced HCC development from 11.7 % in controls to 2.5 % in the treatment groups. In subgroup analysis the protective effect applied to patients with cirrhosis (3.9 % in treated patients and 22.4 % in controls), patients without cirrhosis (1.8 % in treated patients and 8 % in controls), and patients with drug resistance (3.3 % in treated patients and 6.4 % in controls).

It is important to stress that although antiviral treatment reduces the incidence of HCC, the risk is not completely eliminated even in the face of adequate viral suppression and undetectable viral DNA [189–191].

16.4.2.2 Surveillance for HCC

Patients with HCC usually present at late stages when curative treatment is not optional. Therefore, surveillance of patients at increased risk for HCC is recommended. Current guidelines recommend screening for HCC in all cirrhotic patients of all etiologies. In addition, non-cirrhotic chronic HBV patients with active hepatitis or family history of HCC should be under surveillance [42].

Surveillance recommendations are based on an RCT by Zhang et al. [192] that compared biannual screening with ultrasonography and AFP to a control group that received no screening. The screened group completed 58.2 % of the screening tests offered. 9 % of patients in the screening group were diagnosed with HCC compared to 7 % in the control group. 46.5 % in the screened group underwent resection versus 7.5 % in the un-screened population. 1-, 3-, and 5-year survival rates were 65.9, 52.6, 46.4 % versus 31.2, 7.2, 0 %, respectively. The benefits of periodic surveillance have been shown in additional studies [193–196].

In the past few years, the efficacy of AFP testing as a surveillance tool has been questioned. A population-based observational study among chronic hepatitis B carriers in Alaska showed benefit in survival with AFP screening [197], while a randomized controlled study in China showed earlier diagnosis of HCC, but without a reduction in overall mortality [198]. Another study in China found that only 6–8 % of cases not previously identified by ultrasonography were detected with AFP [199], and a later meta-analysis by Singal et al. [200] found no additional benefit to ultrasonography at all with AFP screening. Thus, it was deemed that AFP testing is suboptimal as a surveillance tool. One of the reasons for limited usefulness is that elevation of AFP levels may reflect viral hepatitis flares as well as HCC development, as was found in a trial among HCV carriers [201]. Another problem is that only 10–20 % of early stage HCCs present with abnormal AFP levels [42].

Due to these reasons, current European and American guidelines recommend periodic ultrasonography alone at 6-month intervals [42, 202]. The European Association for the Study of the Liver (EASL) guidelines recommend considering AFP testing when cost is an issue or ultrasonography is not available. The Asian Pacific Association for the Study of the Liver (APASL) guidelines continue to recommend both AFP screening and ultrasonography [203, 204].

A major obstacle in HCC screening lies with patient adherence. A community-based study in California found that 40 % of patients received poor or no screening, with worse screening in non-cirrhotic patients, possibly due to more regular clinic visits among cirrhotics [205]. A systematic review identified nine studies with a pooled surveillance rate of 18.4 %, with better rates among patients followed-up in gastroenterology clinics compared to primary care clinics (51.7 versus 16.9 %, respectively) [206]. Explanations offered for under-surveillance include lack of provider recommendations and failure to identify at-risk individuals [207]. These issues should be the first targets when attempting to improve patient surveillance.

16.4.3 Tertiary Prevention

When patients with chronic HBV develop HCC, they undergo treatments based on tumor staging. However, without elimination of the virus, new HCCs may develop de novo or recur in one or more sites in the liver. Even after successful curative therapy, the majority of patients (excluding transplanted patients) eventually die of multi-focal intrahepatic HCCs and/or of metastasis [208, 209].

Recurrence of HCC is differentiated to two groups—early recurrence (<2 years after surgery) and late recurrence [210]. Variables associated with early recurrence are microvascular invasion and non-anatomical resection. Variables associated with late recurrence are higher grade of hepatic inflammatory activity, multiple tumors and higher viral load, suggesting de novo mechanisms [211, 212]. And so, tertiary prevention in HBV patients aims to reduce late recurrence by decreasing viral load and inflammation [213].

Several studies examined the effects of IFN treatment on HCC recurrence, with conflicting results [151].

With the arrival of NUCs, the survival of patients that underwent resection of HCC, including those with untreated HBV diagnosed with small HCCs, has significantly improved. A study by Piao et al. [214] compared 30 patients after HCC treatment (by different modalities) that received LAM to 40 matched controls that did not receive antiviral treatment. The LAM-treated patients had improved Child-Pugh scores while no such improvement was seen in the controls. There was no

difference in recurrence of HCC, but a significant improvement was seen in liver function and in mortality due to liver failure in the LAM-treated group. Similar results were seen in a retrospective study by Kuzuya et al. [215]. These studies attributed the longer survival of LAM-treated HCC patients to improvement of liver functions. Other studies demonstrated improved tumor-free survival as well [216–220]. Zhou et al. [221] conducted a meta-analysis to assess the impact of HBV DNA levels and NUC therapy on HCC recurrence after resection. Twenty studies were included, and pooled analysis showed that high viral load was significantly associated with risk of recurrence, poorer disease-free survival, and poorer overall survival. NUC therapy significantly decreased the recurrence risk (RR: 0.69, $p < 0.001$) and improved both disease-free survival (RR: 0.70) and overall survival (RR: 0.46).

16.5 Summary

HBV remains a major cause of liver disease, cirrhosis and HCC, especially in Asia and Africa. Molecular mechanisms implicated in HBV-related HCC include direct viral hepatocarcinogenesis and indirect effects via chronic fibrosis, cirrhosis and inflammation.

For HBV-related HCC, primary prevention of HCC is vaccination for all uninfected individuals. Secondary prevention of HCC focuses on those who are already infected and is aimed at suppression of viral replication, and improving surveillance, thus enabling curative treatments. Future treatments to eradicate the virus in chronic carriers will potentially further reduce the incidence of HBV-induced HCC. Tertiary prevention targets patients with HCC and aims to improve survival and reduce recurrence.

Better understanding of molecular pathways involved in HBV-induced carcinogenesis suggests that we are now on the verge of designing new anti-HBV/HCC drugs that will target these pathways, thus reducing the incidence of this deadly cancer.

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Hepatitis C and Hepatocellular Carcinoma

17

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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
DAA	Direct-acting antiviral
SVR	Sustained virologic rate
US	Ultrasonography
CT	Computed tomography
NASH	Nonalcohlolic 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

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Contents

17.1 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.

17.2 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 17.1). 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 coinfection, 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 coinfection [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.

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 non-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 1960s. At that time, the supply of blood for transfusion in Japan was dependent upon 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 upon 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 versus 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].

Genotyping HCV has been important for at least two major reasons in clinical practice: from an epidemiological perspective and because of the predictive value in antiviral 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, determines 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

Table 17.1 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 Ta (Peaked in 1950s–1960s)
Prevalence	0.8 %	1.5–2.0 %
Etiology in HCC	10–15 %	75–80 %

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.

17.3 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-kilobase genome containing a large open reading frame encoding a polyprotein precursor of around 3000 amino acids and untranslated regions (UTRs) at the 5′- and 3′-ends of the genome (Fig. 17.1). 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 17.2) [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 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

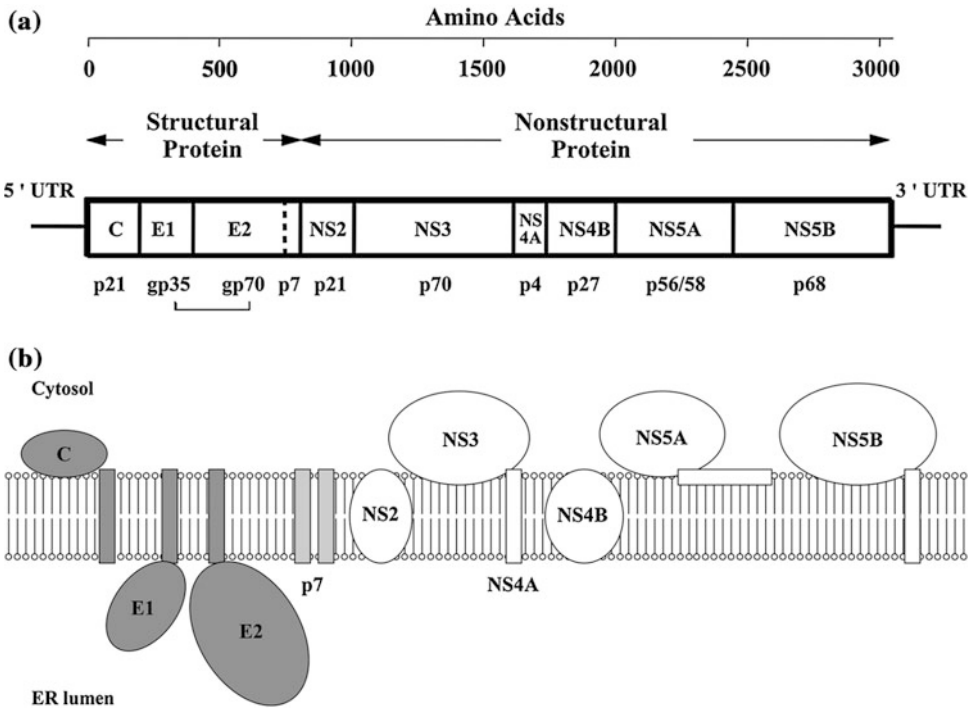

Fig. 17.1 **a**, **b** Structure of hepatitis C virus

Table 17.2 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

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.

17.4 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 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 interferon (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 (SVR) [34, 35]. The resolution of cirrhosis was also noted following a SVR [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.

In the recent progress of direct-acting antiviral agents (DAAs) against HCV, IFN-free treatments are now available for compensated or decompensated cirrhosis [37–39]. DAAs combination therapies now offer SVR rates greater than 90 % for treatment-naive and experienced patients with genotypes 1 through 4. In patients with compensated cirrhosis, sofosbuvir-including regimens for 12 weeks could lead to more than 90 % SVR rates [40]. Recent studies showed the usefulness of sofosbuvir plus ledipasvir for 12 weeks against HCV genotype-1 patients with decompensated cirrhosis. In patients with cirrhosis and moderate or severe hepatic impairment, 86–89 % SVR12 rates were achieved [38]. These treatments have less adverse events during therapies or shorter duration of treatment than IFN-including treatment. Limitations still exist in the current agents, with suboptimal outcomes for genotype 3 and limited data in genotypes 5 and 6.

Eradication of HCV could bring better reserve liver function in patients with cirrhosis and HCV infection although it is unknown whether the occurrence or recurrence of HCC would be reduced in cirrhotic patients [41]. Further studies are needed.

17.5 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.

17.5.1 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, 42, 43]. 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 % [44, 45]. 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, nonalcoholic 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 noncarriers [46]. Among HBV carriers, HBe antigen-positive patients are at a higher risk of HCC than HBe antigen-negative patients (relative risk, 6.3-fold) [47, 48]. 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 [49]. 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 17.3) [34, 50], and chronic hepatitis C patients with cirrhosis have a very high risk of HCC [51]. In European countries and United States, annual incidence rate of HCC is reported to be 0.5–5 % [52]. The reason of this difference is not well known, but maybe related to the difference in the age of patients. Ethnic difference maybe also involved. In Japan, HCV infection spread nationally mainly in the 1950s and 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, 53]. In addition to the degree of liver fibrosis, male gender, older age, and heavy alcohol consumption are the known risk factors for HCV-related HCC.

Cirrhosis due to etiologies other than chronic viral hepatitis also confers a risk of developing HCC. Major etiologies include alcoholic liver disease and NASH [54–56] whose relative importance may differ geographically. Schoniger-Hekele et al. [57] 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 [58]. NASH is a chronic liver disease that is

Table 17.3 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)

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 [59, 60], 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 noninvasive evaluation of liver fibrosis is one of the main areas of interest in hepatology.

One such noninvasive method, transient elastography, correlates well with the histological stage of liver fibrosis [61–65]. The reported cut-off 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 [66]. 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) [67]. 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 [68]. 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 nonnegligible 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.

17.5.2 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.

17.5.2.1 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 [69]. Since 1968, AFP has been used as a serum marker for human HCC [70]. As a marker, AFP reportedly has a sensitivity of 39–65 %, a specificity of 76–94 %, and a positive predictive value of 9–50 % [71–76]. 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 upon the cut-off 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 cut-off 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 cut-off 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 which AFP testing allowed the detection of tumors at an earlier, treatable stage [77]. 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.

17.5.2.2 US

US became available for identifying intrahepatic lesions in the early 1980s [78]. This imaging modality is appealing because it is almost completely noninvasive. 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 % [79], depending upon 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 % [80], 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 [81]. 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 [80].

Although these data are rather disappointing, other reports indicate that the detectability of intrahepatic nodules with US is almost comparable to that of CT [82–85]. In a study of nodules that were ≤ 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 [86]. Overall, US is indispensable in the screening of HCC, as it is noninvasive and less expensive. However, the definitive diagnosis of HCC depends upon 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.

17.5.2.3 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 cut-off values. HCC screening via combined US and AFP may lead to improved detection, although previous reports have been generally negative [72, 87–89]. However, in a nonrandomized 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 [87].

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 [90]. More cases of HCC were diagnosed in the screened group than in the non-screened group (86 vs. 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 of age 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 [91].

17.5.2.4 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 [92]. 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.

Although recent developments identifying serum markers for HCC hold great promise, advances in genomic analysis propelled by new techniques for high-throughout sequencing are likely to further advance the field [93]. Totoki et al. demonstrated the feasibility of sequencing the entire genome of a primary hepatitis C virus-induced HCC [94]. This analysis identified novel mutation patterns and chromosomal abnormalities. Studies such as this will identify specific targets likely to prove useful in both the detection and treatment of 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 [95]. 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 [96, 97]. 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 [98]. 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.

17.6 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 [99]. 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.

17.7 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 noninvasive 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 [100]. 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 cut-off 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.

17.8 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 [101]. 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 [75]. 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 [101]. 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 upon the screening modality used [102].

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.

17.9 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 % [103, 104]. 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 [105]. 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. Recently, the number of HCC patients undergoing resection after IFN therapy has increased. Kubo et al. evaluated the tumor-free and cumulative survival rates for patients who underwent IFN therapy before and/or after curative resection of HCC [106]. The tumor-free and cumulative survival rates of patients who showed a SVR or biochemical response (BR) were significantly higher than those of patients who were classified as nonresponders or who did not undergo IFN therapy. The proportion of patients who died of HCC was significantly lower in the SVR/BR group than in the NR/non-IFN group. In addition, neither SVR nor BR patients died of decompensation. HCV antiviral medications already cure more than 90 % of the HCV population including patients with HIV-HCV, decompensated cirrhosis, and posttransplant [38, 107, 108]. Thus, in patients who undergo liver resection for HCV-related HCC, long-term survival can be expected if antiviral therapy is further improved.

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 [109] 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 [110, 111]. The prevention of the recurrence of HCC, or tertiary prevention, is currently one of the most challenging tasks in hepatology.

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Obesity, NASH, and HCC

18

Chadia L. Robertson, Devanand Sarkar, and Arun J. Sanyal

Contents

18.1 Introduction

Nonalcoholic fatty liver disease (NAFLD) describes a spectrum of liver diseases with pathology resembling liver damage induced by alcohol abuse but occurs in individuals who consume little or no alcohol. Histologically, the scope of NAFLD ranges from instances of simple fat accumulation in the liver to nonalcoholic fatty liver (NAFL) with isolated steatosis and mild nonspecific inflammation, to nonalcoholic steatohepatitis (NASH) [1–3]. NAFL is largely considered to be a benign condition whereas NASH is considered the more progressive subtype of NAFLD often characterized by diffuse hepatocellular injury which can progress to show evidence of necroinflammation, cirrhosis, and fibrosis and in some instances advance to hepatocellular carcinoma (HCC). NAFLD represents approximately 47 % of chronic liver diseases in the US surpassing hepatitis B, hepatitis C, and alcoholic liver disease as the fastest growing cause of chronic liver disease in adults [4]. NASH-associated cirrhosis is the third most common cause of death in NAFLD patients and is predicted to surpass alcoholic liver disease and hepatitis C virus (HCV) to become the leading indication for liver transplantation in the U.S. over the next decade [5].

18.2 Incidence/Prevalence/Risk Factors

NAFLD was first described in 1980 and has since become the most common cause of chronic liver disease worldwide. Few studies include long-term follow up of NAFLD patients. Thus the exact natural history of NAFLD is difficult to ascertain. The global prevalence of NAFLD is however rapidly increasing over time and is currently assumed to range from 20 to 45 % in Western countries and 5–18 % in Asia depending on the studied population and method of diagnosis [5–8]. In the United States, NAFLD is thought to affect approximately 34 % of adults and 20 % of children

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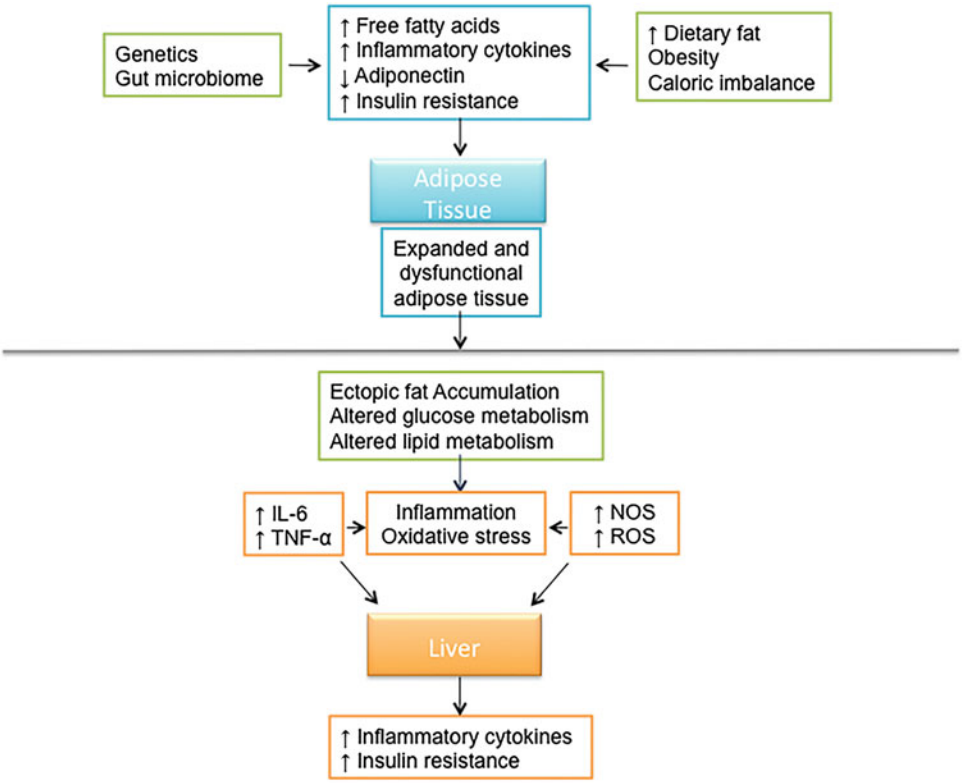

Fig. 18.1 Systemic and liver specific mechanisms involved in the pathophysiology of NAFLD

with an incidence running in parallel to the increased incidence of obesity and diabetes [7, 9, 10].

The global incidence of NASH is estimated to be between 3 and 5 %. Few noninvasive modalities exist which can differentiate NAFLD from NASH. However, population-based studies surveying levels of aminotransferases indicate that NASH affects between 6 and 8 % of adults in the US [11]. A recent study suggests that the frequency of NASH varies significantly with ethnicity with significantly higher incidence in Hispanics (58.3 %) compared to Caucasians (44.4 %) and African Americans (35.1 %) [7]. A close link has been identified between the metabolic syndrome and NAFLD. The metabolic syndrome can be defined by the presence of three or more of the following conditions: visceral obesity, hypertension, type 2 diabetes or elevated fasting plasma glucose, or dyslipidemia including hypertriglyceridemia or low high-density lipoprotein levels. Thus many consider NAFLD to be the hepatic component of the metabolic syndrome (Fig. 18.1).

18.2.1 Obesity

The worldwide incidence of obesity has been rapidly progressing and is now described by the World Health Organization as a global epidemic. Recent studies suggest that there are 1.6 billion overweight and 500 million obese adults globally [12]. The prevalence of NAFLD is increased up to 80–90 % of obese adults and 60 % in hyperlipidemic adults [1, 13]. Approximately 69 % of adults and 32 % of children in the United States are currently considered overweight or obese. This translates to close to 100 million possible cases of NAFLD in the US alone [14, 15]. Recently, a prospective study reported improved clinical, metabolic, and biological outcomes in patients one year after bariatric surgery [16]. Following bariatric surgery, NASH had disappeared in 85.4 % of the study group suggesting that the deleterious metabolic effects of NASH may in fact be reversible [16].

18.2.2 Insulin Resistance

Insulin resistance has been identified with an astounding frequency in individuals with NAFLD and is now thought to play an integral role in its pathogenesis. There appears to be a direct correlation between the degree of insulin resistance and the severity of NAFLD in patients and higher serum insulin levels are found in patients with NASH and fatty liver [17–19]. Furthermore, insulin resistance is consistently found in subjects with NAFL or NASH, even in the absence of diabetes [20]. Similar levels of insulin resistance have

been observed in both overweight and lean patients with fatty liver suggesting that insulin resistance, not simply excess body fat, is essential to the pathogenesis of fatty liver disease [19].

The primary functions of adipose tissue are to store lipids, which can be burned to meet the energy needs of the body and to protect from excesses in circulating glucose by storing triglycerides produced by the liver from sugars. Ectopic fat accumulation describes the scenario wherein lipid accumulation has occurred in a site other than adipose tissue such as the liver, pancreas, or other organs not designed to accommodate excessive lipids loads [21, 22].

The two primary issues that induce ectopic fat accumulation are (1) an excess in energy intake as compared to expenditure, and (2) defects in mechanisms that control the proper shuttling of excess energy as lipids to adipose tissue. NAFLD is an example of ectopic fat accumulation. Hepatic lipid accumulation creates an insult to the liver which induces increased secretion of hepatokines, increased gluconeogenesis, decreased glycogen synthesis, and inhibition of insulin signaling [21, 23]. Excess fatty acids not only induce hepatic insulin resistance but also impair insulin clearance [24, 25].

18.2.3 Diabetes

Type 2 diabetes (T2DM) and NAFLD are closely associated and NAFLD incidence is elevated in 69 % of patients with type 2 diabetes mellitus. Studies show that type 2 diabetics have a 2- to 4-fold increase in serious liver disease and are at increased risk of mortality from cirrhosis, and hepatocellular carcinoma [26–30]. Family history of diabetes and or insulin resistance also increases the risk of cirrhosis and fibrosis in diabetics and nondiabetic NAFLD/NASH patients alike [31, 32]. It has been documented that sustained elevation of plasma FFA levels over time can impair insulin secretion in lean, nondiabetic subjects who are genetically predisposed to T2DM [25]. A convincing body of evidence exists in support of the link between NAFLD and T2DM. Most estimates of T2DM in NAFLD have been based largely on medical history or the less sensitive plasma fasting glucose or A_{1c} levels. Therefore, there is a need for well-controlled long-term prospective studies on the natural history of NAFLD in T2DM using more accurate methods of analysis.

There are also data suggesting that hypothyroidism, hypopituitarism, hypogonadism, sleep apnea, and polycystic ovary syndrome independent of obesity are important risk factors for the incidence of NAFLD [33–41]. Further investigation is warranted to determine if each of these factors truly influence the natural history of NAFLD or exist simply as comorbidities.

18.3 Pathophysiology

18.3.1 Disease Progression

Fatty liver (steatosis) is the more common subtype of the fatty liver diseases and has long been considered as benign. NASH, seen in 10–25 % of NAFLD cases, has been considered the more progressive disease state. Recent findings have prompted a shift in this paradigm. Where previous studies reported that NAFL may be benign, with little to no risk for progression to a more advanced disease, more recent studies provide evidence that progressive fibrosis can develop in both NASH and NAFL patients [42, 43]. In addition, it must be considered that NAFL can progress to NASH with fibrosis indicating that NAFL must also be considered a progressive disease [43]. Estimating the true incidence of NAFLD including NASH has been extremely challenging for many reasons including variability within study groups and lack of accurate noninvasive diagnostic techniques. Tracking the progression from one NAFL to NASH remains a challenge and the factors which may potentially cause progression from NAFL to NASH are still largely unknown.

The progression from non alcoholic steatohepatitis to cirrhosis and advanced fibrosis is a process that has been frequently studied [44–46]. NASH is a complex disease characterized by hepatocyte ballooning, macrovesicular steatosis, inflammation, and pericellular fibrosis. 15–20 % of NASH cases progress to cirrhosis and the 5-year incidence of HCC in individuals with cirrhotic NASH is approximately 11 % [47–49]. Hepatic fibrosis develops in 40–50 % of patients with NASH [27]. The presence of advanced hepatic fibrosis is a key contributor to the development of HCC and a key predictor of all-cause and disease-specific mortality in NASH patients [50, 51]. Therefore, the presence of NASH may in and of itself be considered a risk factor for hepatocellular carcinoma. Recent studies indicate that the metabolic impact of obesity in nonalcoholic fatty liver disease may vary widely even among patients with a similar body mass index (BMI) [52, 53]. Therefore when describing the pathogenesis of NAFLD, it is important to consider that numerous factors contribute to its onset and progression. Based on current research, the disease can be attributed to any combination of genetic, dietary, inflammatory, and environmental factors (Fig. 18.2), which will be further discussed here.

18.3.2 Molecular Mechanisms

18.3.2.1 Contribution of Insulin Resistance

There appears to be a direct correlation between the degree of insulin resistance and the severity of NAFLD. It is now

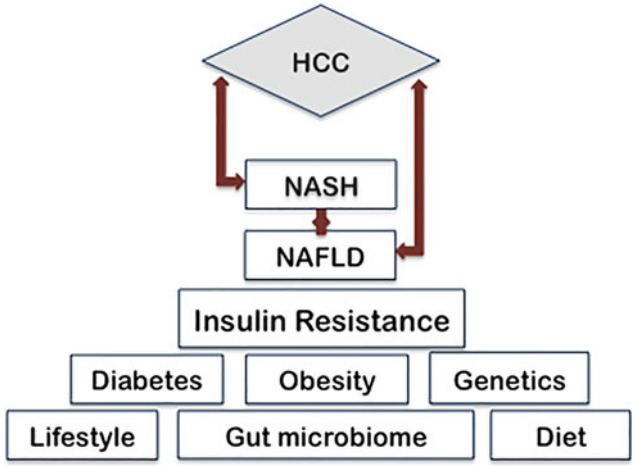

Fig. 18.2 Precursors and modifiers that contribute to the onset of NAFLD and its progression NASH and HCC

clear that adipose tissue dysfunction and inflammation play an integral role in the insulin resistance associated with NAFLD pathogenesis. Adipocytes protect the body from excess energy supply and excess ectopic triglyceride accumulation by activation of several inflammatory pathways. The activation and infiltration of adipose tissue macrophages incite adipocyte dysfunction, adipose tissue insulin resistance, release of excess free fatty acids into the circulation, and ectopic fat deposition [54, 55]. There are two distinct classes of macrophages, which include: The "classically activated" (M1) macrophages and the "alternatively activated" (M2) macrophages. M1 macrophage activation is an essential component of humoral immunity stimulated by microbial products, and proinflammatory cytokines (ex. IFNγ, TNF α). Upon activation, M1 macrophages secrete large amounts of proinflammatory factors including nitric oxide (NO) and reactive oxygen species intermediate (ROI) in addition to numerous proinflammatory cytokines (TNF-α, IL-1, IL-6, IL-12) [56]. By contrast, alternative/M2 macrophage activation is characterized by little to no secretion of proinflammatory cytokines, increased secretion of antiinflammatory cytokines, and enhanced scavenging of cellular debris [56, 57]. Stimulated by the presence of IL-4, -10 and 13, the M2 macrophage response is often associated with tissue remodeling and repair. Obesity generates a state of low grade inflammation and adipose tissue macrophage infiltration often associated with insulin resistance [58, 59]. A systemic increase in the number of M1 relative to M2 macrophages is characteristic of human obesity and animals fed a high-fat diet [54, 57].

Insulin resistance develops when macrophages invade visceral adipose tissue stimulating an inflammatory cascade that includes adipokine secretion [60]. Adiponectin and leptin are adipokines that decrease insulin resistance, while TNF-α, IL-6 and resistin, enhance insulin resistance. Reduced levels of adiponectin and elevated TNF-α and IL-6 are often synonymous with the NAFLD phenotype. Factors implicated in the initial genesis of adipose tissue inflammation, include relative ischemia and production of the hypoxia inducible factor-1, specific gut microflora, and microflora-dependent inflammatory responses and hormones such as leptin [60]. The combination of high circulating insulin levels and high plasma FFAs stimulates hepatic sterol regulatory element binding protein 1c (SREBP-1c) which in turn induces hepatic lipogenesis and oversecretion of very-low-density lipoprotein (VLDL) [24, 25, 61]. Increased lipid synthesis results in increased production of intermediates. By-products of this process include di-acylglycerols (DAG), di-palmitoyl phosphatic acid (Di-P PA), and ceramides [62–65]. DAG in particular is a known contributor to hepatic insulin resistance and is also involved in promoting hepatic inflammation [66, 67]. Elevated hepatic VLDL secretion lowers high-density lipoprotein levels and increases intrahepatic triglyceride accumulation [25, 61, 65]. Together these factors contribute to both chronic liver inflammation and hepatic insulin resistance. Several signaling pathways are known to be involved in this response. The c-Jun N-terminal kinase/activator protein 1, cyclic adenosine monophosphate responsive element binding protein H (CREB-H), the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, and the nuclear factor κB (NFκB) pathways have been implicated in this process [68, 69]. These pathways are activated in response to elevated levels of fatty acids and lipid by-products. Excess in energy intake as compared to expenditure, as observed in obesity, may expose cells to toxic lipids, thereby activating cellular stress pathways. In addition, saturated fatty acids are known to disrupt endoplasmic reticulum (ER) homeostasis inducing ER stress and apoptosis in hepatocytes [70, 71]. This type of cellular stress originates from the accumulation of unfolded or misfolded proteins in the ER and often prompts an adaptive response including activation of the aforementioned pathways ultimately resulting in the release of reactive oxygen species and proinflammatory cytokines such as TNFα and IL-6 [54, 57, 60]. The metabolic consequence of this state is recognized as insulin resistance. Taken together it is clear that in addition to the hepatic milieu there is also a systemic syndrome. As such, the adipose tissue dysfunction and subsequent adipose tissue macrophage activation precede Kupffer cell activation.

18.3.2.2 Contribution of the Gut Microbiota

Several studies have provided evidence suggesting that dysbiosis of the gut microbiota may play a significant role in regulating intrahepatic metabolic and inflammatory pathways that contribute to the development and progression of

NAFLD. The mechanisms responsible for this process are not completely understood but the increased intestinal absorption of multiple bacterial products, such as short-chain fatty acids, lipopolysaccharide (LPS), and endotoxins are thought to be involved [72]. Studies examining fecal microbiota in NAFLD and NASH patients have yielded interesting results. These studies reveal that the microbiota of patients with NAFLD or NASH have a lower proportion of members of the Ruminococcaceae family than healthy subjects [73–75]. Studies have shown that NASH patients have a higher prevalence of small intestinal bacterial overgrowth with elevated expression of TLR-4 and release of IL-8 [76]. A link between the percentage of gram-negative bacteroidetes and the presence of NASH has also been identified [77]. Furthermore, an increase in the abundance of alcohol-producing bacteria has been observed in NASH patients suggesting that these strains in particular may play a role in NASH pathogenesis [75]. Intestinal permeability and bacterial overgrowth correlate with severity of steatosis, but not fibrosis or hepatic inflammation. However, sustained exposure to these inflammatory mediators does promote the generation of various profibrogenic and apoptotic factors [78].

Changes in bacterial metabolites have been associated with obesity, and fatty liver disease. Notably, deficiency in the metabolite choline has been implicated in the pathogenesis of NAFLD and NASH. Diets high in fat and cholesterol promote the formation of intestinal microbiota that converts dietary choline into methylamines [79]. This process results in reduced circulating plasma levels of phosphatidylcholine. Phosphatidylcholine is required for assembly and secretion of VLDL and without it an accumulation of triglycerides is inevitably observed in hepatocytes [79]. Many of the complications of cirrhosis such as hepatic encephalopathy and infections have been linked to dysbiosis of the intestinal microbiome. Increased levels of endotoxin, systemic inflammation, and production of bacterial by-products such as ammonia contribute to pathogenesis. The products of hepatocyte injury and the cytokine milieu combine with systemic factors to promote inflammation within the liver creating a clear progression from fatty liver disease to steatohepatitis. The identification of pathogenic pathways linking the status of the gut to liver function has been eye opening. It is believed that these pathways are driven by dietary changes that could possibly induce gut dysbiosis potentiating hepatic inflammation and ultimately promoting hepatocarcinogenesis [77, 80, 81].

18.3.2.3 Contribution of Genetic Factors

The progression from NASH to end-stage liver disease, i.e., cirrhosis, and HCC is relatively infrequent. This suggests involvement of genetics factors influencing variables such as; hepatic innate immune function, lipid metabolism, extracellular matrix architecture, and cellular transformation resulting in the onset and progression of liver disease. Genome-wide association (GWAS) and candidate-gene studies have provided invaluable insights into the genetic contribution to NAFLD pathogenesis. The patatin like phospholipase 3 (PNPLA3) or adiponutrin gene was the first *bona fide* NAFLD-related gene to be identified using such methods [82, 83]. Individuals harboring the rs738409 C > G single-nucleotide polymorphism (SNP), encoding the Ile 148Met variant protein of PNPLA3 more frequently develop NASH [82]. The rs7384 mutation of PNPLA3 is not only associated with NASH, but also with the severity of necroinflammatory changes independent of metabolic factors and fibrosis [84, 85]. Subsequently, carriers of this mutation are at a threefold higher risk for NASH and 12-fold greater risk of HCC in comparison to noncarriers [86, 87]. The mechanisms underlying the role of PNPLA3 in liver disease are not well understood however the expression of the rs738409 variant is thought to interfere with lipoprotein export shifting the balance in favor of lipogenic activity over lipase activity, leading to hepatic fat accumulation [88–90]. Modifications of PNPLA3 remain the most verified genetic factor in the progression of NAFLD however the contributions of other genetic factors have been described.

Of note, a multi-ancestry, population-based exome-wide association study recently identified a nonsynonymous SNP in the transmembrane 6 superfamily member 2 (TM6SF2) gene producing a glutamate to lysine amino acid substitution at residue 167 (Glu167Lys) [91]. The TM6SF2 rs58542926 SNP (c.449 C > T, p.Glu167Lys) SNP is associated with increased hepatic triglyceride content and is highly conserved across mammals [91]. The TM6SF2 variant encoding p.Glu167Lys results in lowering of the levels of low-density lipoprotein cholesterol (LDL-C), triglycerides, and alkaline phosphatase in 3 independent populations. Carriage of the TM6SF2 minor allele is associated with NAFLD in general and advanced hepatic fibrosis/cirrhosis in particular and thus with increased risk of progression to NAFLD–HCC [92]. Most convincing perhaps was the reported gene-dosage effect, wherein the incidence of NAFLD increased with the number of minor alleles possessed [92]. The fact that hepatic triglyceride accumulation has not been directly linked to hepatotoxicity indicates that more research is required to determine the exact mechanism through which TM6SF2 drives NAFLD-associated hepatic fibrosis.

18.4 Diagnosis

Non alcoholic fatty liver disease is largely asymptomatic particularly in its early stages. In some instances, patients report nonspecific symptoms such as fatigue and fewer still, report pain in the right upper quadrant. Currently, there are

no defined symptoms of NAFLD or physical examination findings which clearly indicate the presence of the disease [93]. Abnormal liver function tests or incidental observations in patients undergoing thoracic and abdominal imaging for reasons other than liver symptoms, often lead to the diagnosis of NAFLD. Three distinct parameters are important for the diagnosis of NAFLD. These factors include evidence of hepatic steatosis by imaging or histology and the absence of competing etiologies for hepatic steatosis or significant alcohol consumption [13]. There are some clinical indicators that have been associated with NAFLD and associated disease. For instance, *acanthosis nigricans* resulting from insulin resistance is often associated with advanced disease, and the presence of a dorso-cervical hump has been linked to nonalcoholic steatohepatitis in some patients [93, 94]. Clinical manifestations including palmar erythema, spider angiomata, gynecomastia, or prominent upper abdominal veins may also be observed in patients following the onset of cirrhosis [93]. Cirrhosis is described as a progressive disease which starts with an initial asymptomatic or compensated phase and progresses to a more advanced decompensated phase marked by portal hypertension and liver dysfunction. Progression from compensated cirrhosis to decompensated cirrhosis is associated with a host of pathologies, such as ascites, jaundice, splenomegaly, and asterixis [93].

18.4.1 Liver Biopsy

Liver biopsy remains the gold standard for identifying patients with NAFLD as it provides a definitive assessment of hepatic steatosis, hepatocellular injury, inflammation, and fibrosis. Numerous limitations are associated with biopsy including patient discomfort, procedure-related complications, sample variability, and observer variability [95]. Despite these limitations, liver biopsy remains the most consistent method of diagnosing and staging NASH. Identification of nonalcoholic steatohepatitis on an initial liver biopsy is a warning sign for the development of liver fibrosis [96]. The progression of liver fibrosis is a key predictor of all-cause and disease-specific mortality in NASH patients [51, 97]. Therefore, early diagnosis of nonalcoholic steatohepatitis and cirrhosis is essential from a treatment and management standpoint. Despite its clear utility, performing liver biopsy on every patient suspected of NAFLD would be impractical and it is thus essential to identify accurate and specific noninvasive methods to diagnose NASH. Several methods currently in use are described in the following section.

18.4.2 Transaminases

While mildly elevated transaminases [alanine aminotransferase (ALT) > aspartate transaminase (AST)] and/or gamma-glutamyltransferase (GGT) may be observed in some patients with NAFLD, over 50 % of patients with advanced disease have normal liver enzyme levels [98, 99]. Additionally, ALT is an unreliable predictor of both steatosis and fibrosis in individual patients [98, 100].

18.4.3 Imaging

Recent innovations in imaging technology have shown potential to change how we both diagnose and monitor liver fat content. Ultrasound is an example of a low-cost, low-risk, and widely available diagnostic tool that may be utilized for qualitative assessment of hepatic disease. In the past, ultrasound was associated with numerous diagnostic limitations including an inability to distinguish NASH from NAFL and poor sensitivity for steatosis below 30 % [101]. A newer quantitative ultrasound technology (QUS) has recently been developed to better characterize tissue microstructure by measuring fundamental acoustic parameters. Improvements on the previous ultrasound technique include the ability to more accurately measure liver fat even in the morbidly obese, and to the ability to identify the presence of steatohepatitis [102]. Potential issues are that results are operator dependent and interpreted qualitatively, therefore open to variability and subjectivity. With continued validation, this method shows promise as a noninvasive method to quantify hepatic steatosis. More studies are required to determine the efficacy of this method in assessing advanced liver disease.

Another imaging modality that can be used to detect hepatic fat is magnetic resonance imaging (MRI), including magnetic resonance spectroscopy. Recent data suggest that magnetic resonance imaging and MRS may be a superior to histological evaluation in assessing longitudinal changes in liver fat content. This method detects the presence of hepatic fat greater than 5.56 % with close to 100 % accuracy [103]. And numerous studies evaluating the diagnostic performance of magnetic resonance MRI modalities for assessing hepatic steatosis and tracking effects of treatments in patients with NAFLD have shown great promise [104, 105]. Unfortunately, though this is both a sensitive and specific method of quantifying liver fat and steatosis, it is also expensive and not widely available. Efforts to increase the availability of MRI modalities will no doubt move us closer to the development of noninvasive determination of NAFLD that identifies the population at risk of worse outcomes and

disease progression, tracks disease progression, and assess response to therapy.

18.4.4 Predictive Models

Several low-cost, noninvasive predictive panels have been developed for the assessment of fibrosis in chronic liver disease. The NAFLD fibrosis (NFS) and fibrosis score 4 (FIB-4) stand out as the most commonly used [106–108]. The FIB-4 score scoring system was originally developed as a predictive measure for advanced fibrosis in HIV patients also infected with hepatitis C [109]. Using this method age, AST, platelet counts, and ALT are evaluated and assessed as predictors of fibrosis. The NAFLD fibrosis score is considered the most validated and best performing predictive panel for evaluation of liver-related outcomes. When calculating the NFS, metabolic risk factors such as age, body mass index, and fasting glucose are evaluated alongside readily available clinical data including platelet count, albumin level, and the ratio of AST to ALT. Thus, NFS may offer a more comprehensive evaluation to identify patients at risk for severe disease. Using the NFS model, advanced fibrosis can be excluded in patients with a score below the low cut-off score of −1.455 (with 75 % sensitivity and 58 % specificity). Conversely, an NFS above 0.676 is an indicator of the presence of advanced fibrosis (with 33 % sensitivity and 98 % specificity) [110–112]. Thus, NFS may be utilized as a low-cost, noninvasive panel to aid in the identification of patients with liver disease who may benefit most from liver biopsy.

18.4.5 Biomarkers of NAFLD

In recent years, a number of biomarkers have been identified which are associated with NASH, such as cytokeratin-18 (CK-18) and terminal peptide of procollagen III (PIIINP). However, no single broadly validated biomarker has been found which can accurately and consistently diagnose NASH.

MicroRNAs (miRNA) are known to play an essential role in a variety of biological processes and have also been implicated in the progression of NAFLD [113]. A number of studies suggest that NAFLD has a distinguishing circulating miRNA profile that may be exploited for diagnostic purposes. MicroRNA-122 is perhaps the most well-characterized liver-associated miRNA as it is the most abundant miRNA found in the liver [114]. Closely linked to metabolic homeostasis, miR-122 has been shown to indirectly modulate the expression of genes involved in hepatic cholesterol and lipid metabolism [115–118]. Studies indicate that serum levels of miR-122 along with miRNAs 192, 375, and 19 were significantly elevated in patients with NAFLD as compared to healthy controls [119–122]. Furthermore, serum levels of miR-122 were shown to successfully distinguish NASH from simple steatosis and to identify liver fibrosis [122]. Based on these studies, circulating miR-122 might be useful as a biomarker for diagnosing fatty liver disease and monitoring the progression of histological changes, during therapeutic intervention.

18.5 Treatment

18.5.1 Lifestyle Intervention

18.5.1.1 Diet

Individuals with NAFLD often have a diet high in saturated fat and cholesterol and may partake in overconsumption leading to energy imbalance and an overweight or obese phenotype. Studies indicate that even relatively moderate weight loss (as low as 10 % of body weight) can improve hepatic insulin resistance and significantly reduce liver fat accumulation [123, 124]. In addition, massive weight loss following bariatric surgery can induce the disappearance of NASH and the partial reversal of cirrhosis in the liver [16, 125, 126]. Thus, it is clear that dietary intervention is an important component in treating NAFLD patients. Caloric restriction drives weight loss, visceral adiposity, subcutaneous fat, and liver fat reduction. Thus, reducing calories appears to be the most significant component of dietary intervention [127]. Also of importance to NAFLD progression, is the quality of dietary fat, as evidenced by the beneficial effects of mono and polyunsaturated fatty acids on fatty liver disease [128, 129]. As such, the Mediterranean diet which is rich in mono and polyunsaturated fatty acids has proven effective in reducing liver fat and improving hepatic insulin sensitivity even in the absence of significant weight loss [130–132].

18.5.1.2 Exercise

It is not entirely clear if exercise exerts independent benefits in patients with NAFLD. The benefits of exercise in improvement of cardiovascular heath and reduction of the risks of the metabolic syndrome are, however, widely known. Fitness affects the response to calorie reduction; thus, improvement of cardiorespiratory fitness may reduce liver fat with diet-induced weight loss [133]. Resistance exercise has been shown to reduce liver fat, improve insulin sensitivity, and promote fatty acid oxidation in NAFLD patients [134]. Improvement of overall fitness may improve the resolution of NAFLD. The intensity and frequency of exercise required to realize improvements in NAFLD is poorly defined; thus, no specific recommendations can be made in this area.

18.5.2 Pharmacological Agents

18.5.2.1 Insulin Sensitizers

Insulin resistance is nearly universal in NAFLD patients and plays an important role in its pathogenesis by inducing peripheral lipolysis, de novo lipogenesis, and ectopic lipid accumulation. Hence, insulin sensitizers make for an attractive target for the treatment of NAFLD. Pioglitazone is the most well-studied pharmacological agent used for treatment of NASH and belongs to the class of drugs known as thiazolidinediones (TZDs). TZDs upregulate adiponectin, promote differentiation of insulin-sensitive adipocytes, enhance fatty acid uptake in adipose tissue, shuttle nonesterified free fatty acids toward adipocytes, and reduce ectopic fat accumulation. Treatment with pioglitazone has been shown to resolve steatohepatitis with the improvement of all individual histological features except for fibrosis. Glitazones use has been associated with a number of side effects, particularly weight gain, which is not always reversible upon discontinuation. Pioglitizone has also been associated with postmenopausal bone loss and instances of congestive heart failure [135, 136].

18.5.3 Hepatoprotective Agents

18.5.3.1 Vitamin E

Vitamin E is a fat-soluble compound that is present in the phospholipid bilayer of cell membranes, the rationale for investigating the use of vitamin E in a NASH patient is based on the role of oxidative stress in NASH progression. Vitamin E is an antioxidant that prevents liver injury by protecting against free radicals and mitochondrial toxicity [137]. Vitamin E has shown moderate efficacy in improving inflammation and ballooning in NASH patients [138]. While generally considered benign, there have been reports of side effects with long-term use of vitamin E which include increased risks of prostate cancer and hemorrhagic stroke [139, 140].

18.6 Conclusion

Nonalcoholic fatty liver disease has become a worldwide heath issue. NASH-associated cirrhosis is the third most common cause of death in NAFLD patients and is predicted to surpass alcoholic liver disease and hepatitis C virus (HCV) as the leading indication for liver transplantation in the U.S. over the next decade. Although NAFLD patients with cirrhosis are at the highest risk of developing hepatocellular carcinoma, we now know that HCC can occur in NAFLD patients in the absence of cirrhosis. Another issue of concern is the increased risk of cardiovascular disease that has been observed in NAFLD patients. Cardiovascular disease is now the leading cause of death in NAFLD patients followed by cancer of the liver. Early detection of patients with nonalcoholic steatohepatitis is of paramount importance if we are to improve patient outcomes through interventional treatment. To that end improvements in diagnostic modalities and drug development are essential.

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Metabolic Disease and Hepatocellular Carcinoma

19

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19.1 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 and 0.90 with increasing tendency in some areas of the world [5, 6] and documents the severity of the disease process once identified [7, 8].

The risk factors for hepatocellular carcinoma vary geographically and include cirrhosis of any cause, chronic hepatitis (especially HBV and HCV [9–11]), toxin induced liver diseases, alcohol and aflatoxin playing the mayor role [12, 13]. While it was not mentioned as possible cause of hepatocellular carcinoma no longer than 10 years ago [14] metabolic liver disease [15] is an increasingly important group to recognize and, as a result, to screen for the development of hepatocellular carcinoma. Furthermore, drugs and other toxins such as pesticides [16] could also lead to development of liver cancer.

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 [12, 17]. 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 [12]. 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 (2–5). 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 [18, 19]. 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 [20]. On the other hand, increased serum insulin levels without the corresponding increase of the c-peptide serum concentration can be the result of the metabolic changes taking place within the liver [21].

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 side and cytokine production and secretion that can result in an enhancement of cellular regeneration on the other side. Moreover, normal control mechanisms that regulate the cell cycle [19] 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 [17].

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.

19.2 Oxidative Stress

ROS and 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 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 (O_2) is converted to singlet oxygen (O^-) by the mitochondria 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 [22]. 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. Under such conditions "pathological polyploidization" may occur; the number of hepatocytes with a single polyploid nucleus may then increase dramatically [23].

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.

19.3 Alcoholic Liver Disease and Hepatocellular Carcinoma

Alcohol consumption is very popular around the world and has become very problematic in younger people in many countries around the world. It can be affirmed that in many countries alcoholic beverages are components of diet. The impact of the consumption of alcoholic beverages in liver diseases can therefore only be roughly estimated. This should be kept in mind when risk factors for liver diseases are considered. In fact, most of the patients presenting with viral liver diseases did not know about carrying the virus until they became sick and of being informed that their life style has to be modified. It also has to be considered that quantification of consumption of alcoholic beverages is totally dependent on the information given by the patients. Every day experience of physician dealing with patients with liver diseases tell us, however, that patients tend to hide the attitude of regular intake of alcoholic beverages. Nevertheless, it has been published that two third of American adults drink some alcohol [13]. While the risk of cirrhosis development increases with a daily intake of more than 30 g/day of alcohol, 10–40 g/day of alcohol is considered to be compatible with the diagnosis of nonalcoholic liver disease (NAFLD) [24]. 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 [25]. 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. Alcohol

consumption may be responsible for a part of liver cancers which develop in patients who eliminated the HCV after successful treatment [26].

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 [27, 28].

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 [29]. Alcohol-induced reductions in tissue folate levels enhance these effects by impairing transmethylation pathways [30]. A reduction in the level of cellular pyridoxaldol 5-phosphate induced by alcohol abuse is also important [31, 32]. Each of these effects results in enhanced DNA hypomethylation and upregulated gene expression particularly of proto-oncogenes and subsequently activated oncogenes [33–36].

DNA methylation occurs predominantly at the fifth carbon atom of cytosine–guanine pairings [37]. This dinucleotide pairing frequently occurs within the promoter region of genes. Hypermethylation silences gene expression while hypomethylation which can occur 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 [38, 39].

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 [40]. 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 [41, 42]. 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 [43, 44]. 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 [45, 46]. 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 [47, 48].

Each of these consequences of alcohol abuse (folate and B6 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.

19.4 Nonalcoholic Fatty Liver Disease (NAFLD), Nonalcoholic Steatonecrosis (NASH) and Heptocellular Carcinoma (HCC)

NASH was described by Ludwig and associates in 1980 [49]. 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 past 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 [50]. NAFLD can progress to NASH which is not an innocuous process but has the potential to progress to cirrhosis with NASH or cryptogenic cirrhosis [51], both of which can develop HCC [52] 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 than that of chronic hepatitis C (HCV) [53]. 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 [54, 55].

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 [56, 57].

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 [58].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 [59]. 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 [60, 61]. 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 [62]. The first hit is most likely an increase in hepatic fat as a consequence of hypertriglyceridemia. The opposite may be the result of insulin resistance. Insulin resistance is known to result in a diffuse reduction in tyrosine phosphorylation [63, 64] 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 [65–68]. Each of these events contributes to a state of considerable oxidative stress [69]. As a result of the combination of lipid peroxidation, the production of 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 manifested.

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 iodine or an intravenous contrast allergy, an annual MRI with an iron-containing contrast agent can be substituted for the triple-phase CT scan.

19.5 Hemochromatosis and Wilson's Disease and HCC

Both iron and copper have the potential to be mutagenic as a result of oxidative stress [70]. An abundance of DNA adducts has been identified in the hepatic tissue of individuals with hemochromatosis and Wilson's disease [71]. DNA damage of hepatocytes exposed to iron has been demonstrated in vitro and most probably also occurs with copper exposure [72–75]. Classic hemochromatosis is an autosomal recessive disorder that occurs at a rate of 1/1000 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.

About 10 % of the C282Y homozygous with serum ferritin levels above 1000 μg/L develop the disease, while those with a serum ferritin level below 1000 μg/L are at low risk of developing hemochromatosis [76]. The low penetrance of the hereditary hemochromatosis phenotype strongly suggests that other factors are crucial for the development of the clinical disease. The synergistic effects between increased hepatic iron storage and other co-factors, e.g., alcohol [77, 78] have to be excluded before specific therapy is started.

While increased mortality has been reported for hospitalized patients with HH homozygous, the C282Y mutation C282Y individuals identified by population screening or among blood donors do not show any reduction of life expectancy. HH-patients identified in an outpatient service, however, are younger and do not show a relevant increase of the mortality risk as it was found to be the case for their relatives [79]. 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 [80].

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 unbalance in all 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 [81, 82].

Hepatocellular carcinoma is reported in 7.5–30 % of cases of hemochromatosis [83–86]. Almost all the cases have been reported in cirrhotics but at least two cases have been reported in noncirrhotics [87]. 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 [84, 85, 87].

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 [72–87].

19.6 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 (624 gt) is found [88].

Aflatoxin is metabolized to a potential mutagenic intermediate, aflatoxin 8, 9-epoxide, which is normally detoxified by microsomal peroxide hydrolyses and glutathione S-transferase [88]. 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 [88].

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.

19.7 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 a 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 [89] and the protein accumulates in the endoplasmic reticulum of the liver [90, 91]. In addition, mitochondria dysfunction and autophagy occur and contribute to the overall hepatic dysfunction and resultant disease progression [92]. 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 [93–98].

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 homozygous 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 together 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.

19.8 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 [99]. 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 [100]. 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 [101]. 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 [102].

(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 [103]. 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 characterized 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.

19.9 Bile Acid Synthetic Disorders and Hepatocellular Carcinoma

Nine distinct genetic disorders of bile acid synthesis have been identified and characterized clinically [104]. 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 [105]. 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 diseases 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 where in the manifestations occur and involve the nervous system, adrenal glands, as well as the liver.

19.10 Defects in Carbohydrate Metabolism

19.10.1 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) [106]. 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 *Escherichia coli* sepsis particularly in neonates. A single report of HCC in a child with this disorder, who had a transplant, has been be treated medically, and if treated appropriately with a galactose-free diet clinical liver disease should not occur [107].

19.10.2 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 [108]. Hepatic adenomas and cancer have been reported in types I, III, and IV [108–112]. Tumor detection in each disorder is dependent on 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-Ib as a result of using frozen tissue that enables the catalytic activity of the endoplasmic reticulum to be assayed and detected 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.

(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 [110–112].

19.11 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 [113]. 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 [114]. The apoptotic signal in tyrosinemia type I appears to be fumarylacetoacetate [114]. 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 [115–121]. 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 [122]. 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.

19.12 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 PCT 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.

(G) (H) 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, and HEP but not in EPP [123–131].

19.13 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 [132].

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 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.

19.14 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 [133]. 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.

19.15 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 [134, 135].

19.16 Fanconi Anemia

This is an autosomal recessive disorder characterized by diffuse congenital anomalies, bone marrow failure, and malignancy [136–139]. 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 tumorigenesis in this disorder.

19.17 Type II Diabetes Mellitus

This is a common disorder accounting for >85 % of all cases of diabetes mellitus 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 [140–144].

19.18 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 [145].

19.19 Hereditary Hemorrhagic Telangiectasia

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 [146].

19.20 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 [147–154].

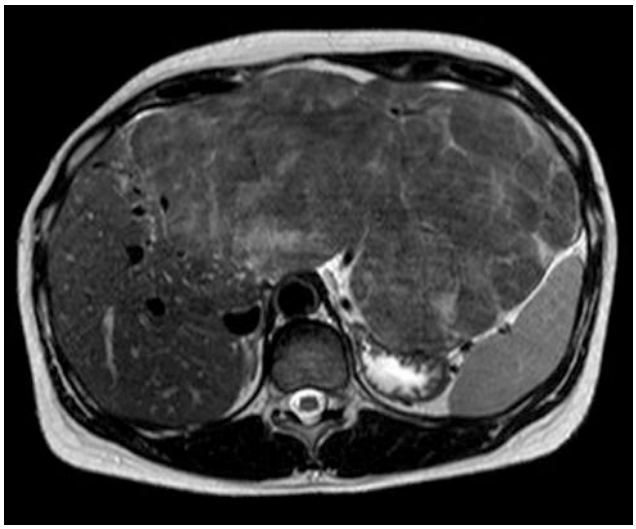

Fig. 19.1 MRI-scan picture of the abdomen of 42 year-old woman who presented persistent pain of the left upper abdomen. The patient was treated with flucotisone twice a day (oral spray) for three years because of pulmonary sarcoidosis. No liver disease was known before this time point. The resected hepatocellular carcinoma (AFP-positive) measured 27 cm and weighted 2825 g. The right lobe of the liver was healthy (kindly provided by Dr. Eleonora Ramadori)

19.21 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 [155, 156].

Androgens are used for their anabolic activity and more often than estrogens produce HCC [157–161].

19.22 HCC in Extrahepatic Chronic Inflammatory Diseases

Occurrence of cases of hepatocellular carcinoma has been described in systemic lupus erythematosus [162], and in systemic sarcoidosis (Fig. 19.1) [163–165] and in Crohn's disease [166, 167] but not in rheumatoid arthritis [168]. In most of the cases, the liver was not affected by the chronic inflammation. Especially, patients with sarcoidosis should be regularly checked for liver cancer development. The role of the therapeutic agents used in those patients in liver cancer development has not been determined so far.

19.23 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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Part II
Diagnosis

Aspects of Hepatocellular Tumor Pathology

20

Michael A. Nalesnik

Contents

20.1 Introduction

The pathologic analysis of hepatocellular carcinoma (HCC) is updated almost daily by advances at the molecular level. However, contextual assessment of these advances is dependent upon the proper diagnosis of HCC and its distinction from other malignant or benign tumors that may involve the liver. Tissue diagnosis is not necessary in every patient, but problematic tumors require pathologic examination for definitive diagnosis; in addition, tissue samples provide a valuable resource for directed studies that may provide prognostic or therapeutic (theranostic) information. In the United States, pathologic evaluation of the explanted liver in transplant recipients previously diagnosed with HCC is mandated by the OPTN/UNOS as a quality control measure to monitor the performance of transplant programs.

This chapter categorizes hepatocellular neoplasms and relevant non-neoplastic growths following established pathologic headings. Detailed molecular analysis is provided elsewhere; however, selected aspects of these features are incorporated into this discussion, particularly in cases where the relevant protein is detectable in tissue and can be exploited for diagnostic, prognostic, or therapeutic purposes.

20.2 Focal Nodular Hyperplasia

20.2.1 Clinical Aspects

Focal nodular hyperplasia (FNH) is a benign mass lesion that may be single or multiple and arises from a hyperplastic

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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 [2]. However, Rifai et al. [4] followed a series of 20 pregnant women with FNH and found no significant growth or complications due to this lesion during this time interval. 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, hereditary hemorrhagic telangiectasia [6] and congenital portosystemic shunts [7]. 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 [8]. Masetti et al. found hematopoietic stem cell transplantation to be an important risk factor in this setting [9]. The radiographic appearance of typical FNH is diagnostic and most cases are detected incidentally during abdominal radiographic examination for other conditions. The lesion may contain fat which in some cases can cause concern for hepatocellular adenoma (HCA) and require biopsy to resolve [10]. FNH is usually a clinically benign condition and in many cases it can be followed without surgical intervention. Rarely, larger lesions can undergo significant hemorrhage [11] or cause other symptoms such as pain [12]. Exceptionally, HCC has been observed to arise within these hyperplasias [13].

20.2.2 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. 20.1). This characteristic feature facilitates radiographic diagnosis in most cases. Variations include eccentric scars and multiple smaller fibrous scars. Importantly from a diagnostic perspective, HCC may on occasion also contain a central scar and must be distinguished from FNH [14].

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 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 HCAs, and they are discussed in that section (below).

Many but not all FNH are solitary and small. In a recent series, 80 % of FNH were under 5 cm, 18 % between 5 and 10 cm, and 2 % greater than 10 cm in diameter [15]. In approximately 20 % of cases, multiple FNH coexist. Composite FNH and HCA has been described [16]. Further, a diagnosis of FNH in one lesion does not ensure that all other lesions are identical, as concurrent HCC may also occur in livers harboring FNH [17, 18].

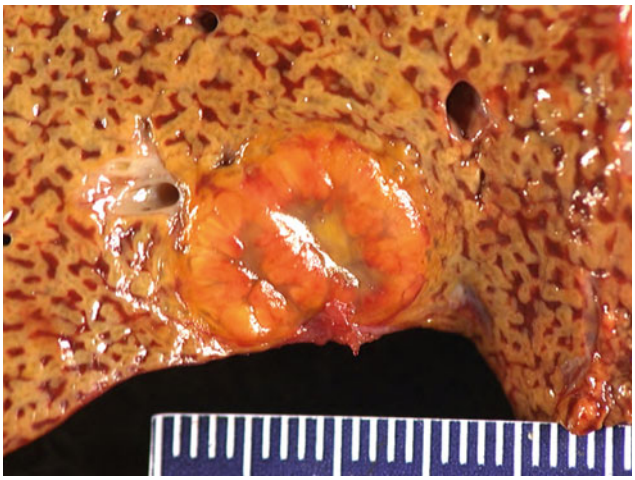

Fig. 20.1 Focal nodular hyperplasia arising in a non-cirrhotic liver. The nodule has centrally depressed areas corresponding to the central fibrous scar. The background liver shows chronic passive congestion

20.2.3 Microscopic and Immunocytochemical Aspects

The microscopic appearance of typical FNH is dominated by architectural distortion produced by a central area of fibrosis from which individual fibrous septa radiate and circumscribe complete and incomplete nodules of normal-appearing hepatocytes (Fig. 20.2). 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. 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 absence of bile ducts that normally accompany 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, possibly related to microenvironmental differences in blood and bile flow within the lesion. The change is similar to the so-called “biliary interface hepatitis”

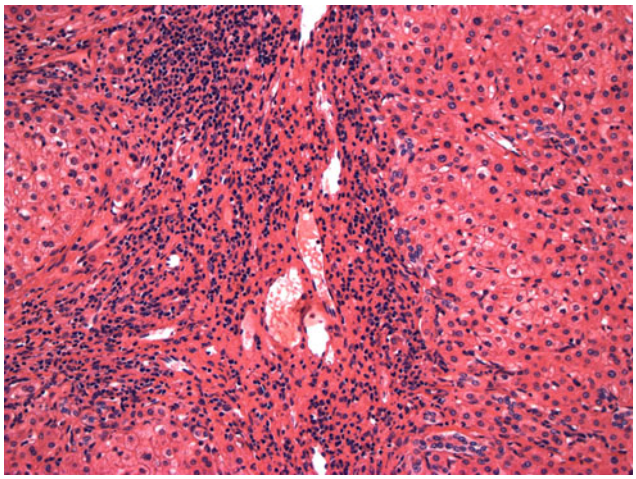

Fig. 20.2 Focal nodular hyperplasia. The *open spaces* in the center represent vessel lumens in a fibroinflammatory area. Proliferative bile ductules are at the interface between fibrous tissue and normal-appearing hepatocytes

seen 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 HCA. We have seen rare examples of the latter condition (as well as HCC) producing a positive copper stain, however, and the diagnosis must take the entire appearance of the lesion into account.

A needle biopsy may be performed for those 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 can 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. 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 diagnoses of HCA or well-differentiated HCC often arise, particularly in needle biopsies in which ductular proliferation is absent. Immunocytochemical stain for glutamine synthetase is often the single most useful stain to distinguish among the alternatives. FNH shows an irregular expansion of glutamine synthetase uptake in a so-called "map-like" pattern. This feature is diagnostic of FNH but can at times be difficult to discern, particularly in small fragmented biopsies where the possibility of a fragment of diffusely staining lesion (suggestive of HCC) may occur. Limitations in the use of this stain have recently been addressed by Joseph et al. [19]. In practice, a panel of stains addressing the possibilities of HCA and HCC are also usually employed as dictated by the histologic appearance and a conclusion can be reached in almost all cases in which an adequate sized tissue sample is provided.

20.2.4 Molecular Aspects

FNH is considered to represent a polyclonal process, although some studies have detected a clonal component [2]. Genetic mutations have not been described.

20.3 Hepatocellular Adenoma

20.3.1 Clinical Aspects

HCA represents a final common pathway of several separate causes of autonomous hepatocyte growth with varying tendencies toward superimposed malignant evolution. Most commonly it is a benign liver tumor arising in women of childbearing age and with a history of oral contraceptive use [20]. In one early study [21], HCA occurred at a rate of 0.1 per 100,000 women per year in the absence of a history of oral contraceptives, rising 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 HCA, and an example of this lesion arising in conjunction with growth hormone therapy for Turner's syndrome has been reported [22]. Use of the antiseizure medication oxcarbazepine has been associated with HCA in mice and in a single recent clinical case report [23]. 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 [24] for low-grade symptoms, feeling of fullness, or other conditions. Intratumoral hemorrhage or rupture with hemoperitoneum may occur, particularly with larger tumors. A study of 124 adenomas found a mean size of 10.5 ± 4.5 cm in ruptured tumors, with no rupture in any tumor less than 5 cm in diameter [25]. However, Bieze et al. [26] more recently

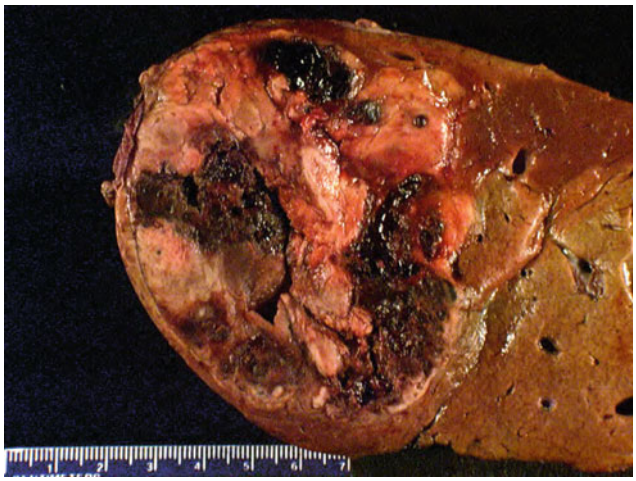

Fig. 20.3 Hepatocellular adenoma arising in a non-cirrhotic liver. This 9.5 cm tumor occurred in a middle-aged woman with a long history of oral contraceptive use. The *dark areas* are due to hemorrhage that led to pain, which represented the presenting symptom of this benign tumor

concluded that the risk for bleeding was increased in tumors 3.5 cm or larger. Additional risk factors for rupture include increasing tumor size, recent hormone use, exophytic growth and location in liver segments II and III [25, 26]. The possibility that HCA may regress if hormonal stimulation is withdrawn has also been noted [27]. An overall 4.2 % frequency of malignant transformation was reported by Stoot et al. in a systematic review of 1617 published cases [28].

20.3.2 Macroscopic Aspects

Hepatocellular adenoma characteristically appears as a well-circumscribed, non-lobulated lesion or lesions within a non-cirrhotic liver (Fig. 20.3). 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 [29]. The term adenomatosis had previously been used to define the existence of 10 or more tumors. However, this is not a discrete condition, but occurs preferentially with certain adenoma subtypes [30] (below). HCAs vary in color from yellow to tan and can be variegated due to a combination of intratumoral hemorrhage, infarction, and fatty changes [31, 32]. The tumors are usually unencapsulated.

20.3.3 Microscopic Aspects

Hepatocellular adenomas are comprised of normal-appearing hepatocytes arranged in a trabecular architecture ranging from one to three cells thick (Fig. 20.4a). There are no normal portal tracts and therefore normal hepatic microanatomical relationships are lacking. The hepatocyte nuclei are small, round, and uniform with inconspicuous nucleoli and few to no mitoses. Cytoplasm can be pale, eosinophilic or steatotic. Cholestasis is not uncommon. Occasionally the tumoral hepatocytes may contain PAS-positive, diastase-resistant hyaline globules [33, 34], Mallory's hyaline [35], or degenerate-appearing hyperchromatic nuclei [36]. 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 occur throughout the tumor (Fig. 20.4). Occasional larger vessels are seen and may also appear as "feeding" vessels adjacent to the tumor. Sinusoids may appear normal, ectatic or even peliotic and on occasion dystrophic blood vessels can be seen [20]. Rarely, extracellular myxoid change without evidence of intracellular mucin has been described [37].

Distinction of HCA from well-differentiated HCC may be difficult or impossible by histology alone. The clinical context is important in this regard, and the diagnosis of hepatic adenoma outside of the setting of a young woman taking oral 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 or to architectural differences such as solid growth or formation of pseudoacini. Micchelli et al. [38] noted cytologic atypia in the form of enlarged and hyperchromatic nuclei as a background change in two of three HCAs harboring foci of HCC. However, background atypia was also observed in several adenomas in which a malignant component was not demonstrated, and the authors concluded that additional studies were necessary to confirm this possible association.

20.3.4 Hepatocellular Adenoma Subtypes and Ancillary Studies

Recognition of specific subtypes of HCAs is based on the Bordeaux classification developed by Bioulac-Sage and associates [39–41]. This system recognizes four categories with distinguishable molecular and immunocytochemical features, partially separable clinical contexts, and differing malignant predispositions.

Inflammatory (inflammatory/telangiectatic) HCAs comprise the most frequent subtype, accounting for at least half of all adenomas. These lesions show variable degrees of inflammation and/or sinusoidal ectasia and may contain a steatotic component. In some cases the presence of a ductular reaction may simulate FNH [2]. They tend to arise in the setting of obesity or alcohol use and may be single or

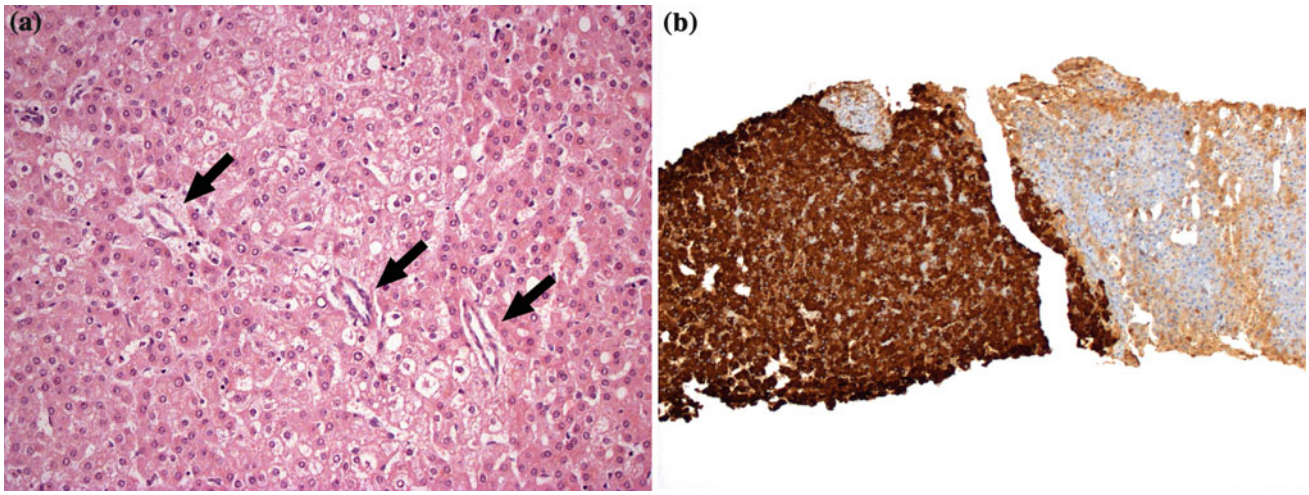

Fig. 20.4 **a**: Hepatocellular adenoma. The tumor is comprised of normal-appearing hepatocytes in unremarkable trabecular architecture. Isolated arteries (*arrows*) are typical but can also occur in other conditions. (200×). **b**. Needle biopsy of separate hepatocellular adenoma immunostained for C-reactive protein. Heavy staining of the adenoma (*left*) with relative sparing of background liver parenchyma (*right*) supported a diagnosis of inflammatory subtype of hepatocellular adenoma (100×)

multiple. Several molecular alterations have been described in subsets of this category, all leading to STAT3 activation. These include mutation of STAT3 itself, activating somatic mutations of the IL6 receptor IL6ST (gp130) [2], JAK1, GNAS, [42] or the src-like kinase FRK [43].

Approximately 10 % of inflammatory adenomas also contain mutations of the β-catenin gene that appears to be a secondary event and is associated with an increased risk of malignant transformation, the extent of which is presently undefined. In one series, 10.6 % of telangiectatic adenomas contained foci of HCC which did not necessarily correspond to β-catenin mutation [44].

Diagnosis of inflammatory HCA is facilitated by the immunohistochemical demonstration of the inflammatory proteins C-reactive protein (Fig. 20.4b) or serum amyloid-associated protein in a diffuse pattern. Stat-3 activation can also be detected by antibody specific for phospho-stat-3.

HNF1α-mutated HCA constitute the second largest class, approximately 35–40 % of tumors. These lesions tend to be markedly steatotic and have a tendency toward multiplicity. Indeed, there may be a family history of multiple hepatic adenomas (adenomatosis). It occurs predominantly in females and there may be a history of diabetes, in particular maturity-onset diabetes of the young, type III. The molecular defect consists of an inactivating mutation of the HNF1α gene and the absence of liver fatty acid binding protein, the product of a target gene of HNF1α, serves as an immunohistochemical marker of this subtype. Bioulac-Sage et al. have observed that the actual number of these tumors in a given patient tends to be underestimated, and small fatty foci in the background liver will also frequently show absence of fatty acid binding protein, indicating that they represent nascent adenomas [39, 40]. Although multiple tumors in this setting most often reflect multiple HNF1α-mutated HCA, rarely they may coexist with other subtypes [45].

HCA with mutations in the β-catenin gene represent the least common defined subgroup, constituting between 10–15 % of HCA. These occur preferentially, although not exclusively, in males and show an association with androgenic steroids, glycogenesis, and familial adenomatous polyposis [40]. This subgroup has the highest risk of evolving into HCC, particularly in males [39]. These HCA do not usually show the steatosis associated with HNF1α-related tumors but are more likely to contain cellular atypia. β-catenin mutation can be evidenced immunohistochemically by demonstrating nuclear translocation of this protein. Diffuse uptake of glutamine synthetase, the protein product of a β-catenin target gene, is also presumptive evidence of this subtype [40] (Table 20.1).

The remaining HCA do not contain evidence of mutations in known associated genes or express inflammatory proteins and likely comprise a heterogeneous group that awaits additional study.

The evolution of HCA into HCC continues to be defined by Zucman-Rossi and associates. In adenomas, an activating CTNNB1 mutation is thought to occur first, followed by telomerase reverse transcriptase (TERT) promoter mutation in conjunction with global hypomethylation and associated chromosomal aberrations as a final step toward malignant transformation, whereas TERT promoter mutation occurs at an earlier stage in the dysplasia to carcinoma sequence that occurs in the cirrhotic liver [46].

20.4 Hepatocellular Dysplasia

Cirrhosis is a preneoplastic condition that provides the background for the stepwise development of HCC through a process of dysplasia. Histopathologic concepts diagramming this progression were put forth in 2009 by an International Consensus Group [47] and our understanding of the molecular events underpinning this sequence has also increased in recent years [46].

The small preneoplastic and neoplastic nodules that can be histologically distinguished include low-grade and high-grade dysplasia, early HCC and classic or progressed HCC. Although these represent sequential steps in the progression to cancer, it is not certain that each nodule will advance to the next stage, nor is it clear that HCC must necessarily pass through all stages.

Low-grade dysplasia is a nodular lesion that can contain portal tracts within its substance, a feature that it shares with the benign large regenerative nodule. However, an increased arterial vasculature that will eventually provide the sole blood source to HCC commences at this stage, evident as occasional unpaired arteries. Hepatocytes remain bland on both a cytologic and architectural basis, though a mild increase in cell density may be seen.

High-grade dysplasia retains features of low-grade lesions such as continued presence of intralesional portal tracts and scattered isolated arteries, the latter likely more frequent on a statistical basis. However, there are now superimposed alterations of the hepatocytes themselves. These consist of nuclear changes such as hyperchromasia and mild irregularities of the nuclear membrane in addition to a variety of potential cytoplasmic changes that may impart a different appearance to the nodule compared to surrounding liver. These can include steatosis, increased or decreased iron deposition, and/or basophilia. The overall nuclear to cytoplasmic ratio is often increased to twice or more that of surrounding liver and the architecture can have features such as pseudogland formation, irregular trabeculae, or intralesional expansile nodules ("nodule in nodule").

These attributes increase in degree with the onset of early HCC and the point at which a set of features qualifies as cancer can be extremely difficult to ascertain. However, one feature that can be used to define the onset of early HCC, which has a macroscopic vaguely nodular appearance, is that of stromal invasion (Fig. 20.5a, b). This refers to the infiltrative presence of lesional hepatocytes within either the fibrous tumor pseudocapsule or within intralesional portal tract stroma. Since the liver is a three-dimensional structure, this must be distinguished from oblique sectioning that may simulate stromal invasion and can be accomplished by the use of cytokeratin 19 stain. The interface of hepatocytes and fibrous stroma typically contains cholangiolar cells that are cytokeratin 19 positive and these cells are lost in the setting of true invasion. Interestingly, some early HCC have extremely well-differentiated tumor cells, such that they may appear on cursory examination to be less worrisome than high-grade dysplastic nodules. This raises the possibility of an alternative process that bypasses typical high-grade dysplasia.

On a molecular level, telomerase reactivation is highly correlated with the development of early HCC [44]. This is due to promoter mutations, HBV insertion into the promoter, or amplification of TERT. Such changes can also be seen in a small minority of low- and high-grade dysplastic nodules. However, the marked rise in frequency seen in early HCC remains stable throughout later stages of this cancer and precedes the multiple genetic abnormalities that characterize individual fully developed HCC. In this regard TERT can be

Table 20.1 Immunophenotypic features of hepatocellular adenomas and other forms of hepatocellular nodules

	FNH	HCA-H	HCA-I	HCA-B	Dysplastic nodule	(Early) HCC
Glutamine synthetase	Positive "map-like" pattern	Focal to absent	Focal to absent, positive if beta catenin mutated	Positive diffuse	Absent to diffuse, not map-like	
Beta catenin (nuclear)	No	No	10 % positive, usually negative	Yes	Variable	
LFABP	Present	Absent	Present	Present	No data	
SAA/CRP	Focal	Focal	Diffuse	Focal	Variable	
Glypican 3	Negative	Negative	Negative	Negative	May be positive; suspicious for HCC	
CD34	Heterogeneous	Heterogeneous or diffuse				Diffuse
CK7	Positive	Negative	Positive (some)	Negative	Negative (most)	
CK19	Positive	Negative	Negative	Negative	Negative	Negative (most)
Phospho-STAT 3	Negative	Negative	Positive (~60 %)	Negative	No data	
Stromal invasion	Negative					Positive

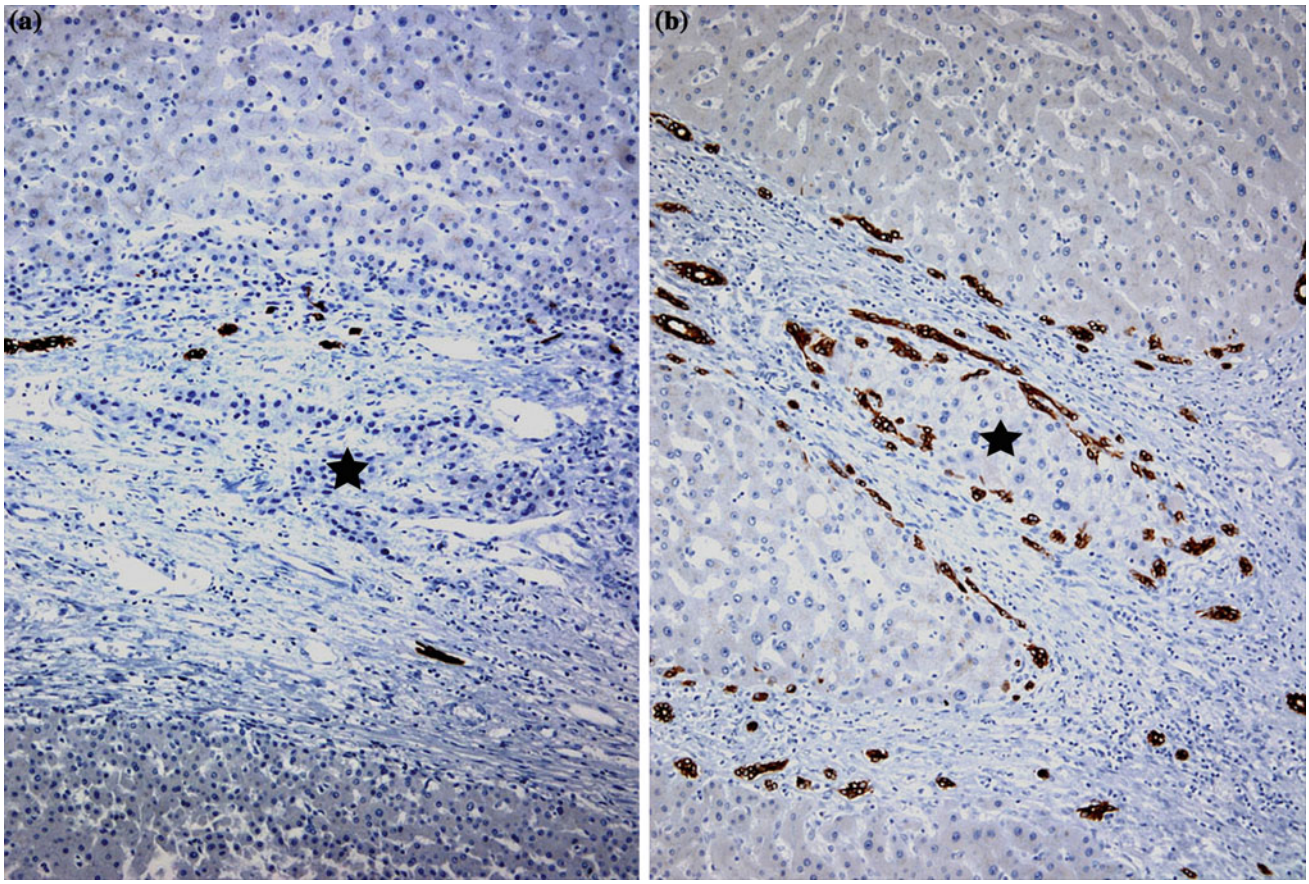

Fig. 20.5 **a**. Stromal invasion. This immunostain for cytokeratin 19 shows a nest of epithelial cells (*star*) within fibrous stroma in the absence of an intervening cytokeratin 19-positive border. The epithelial cells represent malignant hepatocytes invading the fibrous stroma, supporting a diagnosis of early HCC. **b**. Representative partial section through a hepatic nodule for comparison. This benign collection of hepatocytes (*star*) represents the edge of a cirrhotic nodule and shows the expected cytokeratin 19 positive peripheral distribution of ductular cells

considered as a gatekeeper gene, with reactivation allowing the cells to escape from senescence and continue to proliferate. As a corollary, early HCC is genetically closer to dysplastic nodules than it is to typical clinically evident HCC.

From a diagnostic standpoint, Di Tommaso et al. [48] described the utility of immunohis- tochemistry in separating hepatocellular dysplasia from early HCC. 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 dysplastic nodules. However, these stains may individually be positive in a minority of dysplastic lesions and diagnosis at present requires attention to both histologic and immunocytochemical features.

Progressed or typical HCC presents no difficulty in diagnosis, appearing as a distinctly nodular lesion and representing a fully developed, albeit small, cancer with all of the features typically described for this tumor.

20.5 Hepatocellular Carcinoma

20.5.1 Clinicopathologic Comments

Approximately 85 % of HCC arise in the setting of cirrhosis, which provides a field effect predisposing to neoplasia. Thus, exposure to agents leading to cirrhosis as well as genetic polymorphisms that may predispose to enhanced injury in an agent-specific manner increase the risk of cancer. Such HCC evolve in a stepwise fashion through the dysplastic process described above. The requirement for cirrhosis and superimposed evolution of dysplasia may account in part for the fact that the median age of diagnosis in the US is 64 years [49]. Greater exposure to common risk factors such as alcohol or hepatitis C virus [50] in males, as well as the reported protective effect of estrogen [51], may contribute to the 3:1 male:female ratio of HCC.

A separate pathway exists via malignant transformation of HCA. High- and low-risk variants exist (above) with direct emergence of HCC in the apparent absence of an intermediate dysplasia process.

20.5.2 Macroscopic Aspects

The majority of HCCs arise in cirrhotic livers and most frequently involve the right lobe. The tumors are typically soft, vary in color from gray-green-yellow to light brown, are occasionally bile-stained or fatty, and often contain foci of hemorrhage or necrosis. Rarely they may contain a central scar mimicking FNH [14]. The tumors can be single or multiple and range from less than 1 to over 30 cm with a tendency toward larger size at diagnosis in the setting of non-cirrhotic liver [52, 53]. A variety of macroscopic patterns exists but has few clinical correlates. The traditional classification of Eggel [54] distinguishes three patterns of HCCs: multinodular, massive, and diffuse. Multinodular HCC is typically associated with cirrhosis and is characterized by multiple tumor nodules scattered throughout the liver [53, 55]. In the massive pattern a solitary tumor mass occupies much of the liver and may coexist with smaller satellite nodules. This pattern has been associated with non-cirrhotic livers. 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 [55, 56]. Rarely, HCC may be pedunculated, possibly reflecting origin from an accessory lobe [57]. In one study it was concluded that pedunculated HCC has an unfavorable prognosis if appropriate surgical procedures are not performed during early development [58].

In more recent macroscopic classifications, HCCs 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 [59].

Portal vein thrombosis occurs in a high proportion of advanced cases [60] with a lower frequency in small HCC [61]. It has been proposed that curative resection may be possible in the presence of portal vein invasion if the primary tumor is small, i.e., early stage [62].

Less frequently, HCC may involve the main hepatic veins, the inferior vena cava or right atrium and it can also extend into the large bile ducts. The clinical consequences of those involvements include Budd–Chiari syndrome, biliary obstruction, and hemobilia [63–66].

Tumor stage is the primary macroscopic determinant of prognosis, and additional visible tumor features provide further information only to a limited degree. For example, a diffuse growth pattern makes 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 [55, 56]. He et al. [67] found the infiltrative growth pattern to be associated with shorter disease-free and overall survival following hepatectomy. Periportal tumor location is a risk factor for recurrence following radio frequency ablation [68] and serosal invasion a risk for recurrence following curative resection in some series.

20.5.3 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 with the most recent update in 2010 [69]. Most 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 or multiple tumors, individually 5 cm or less in size. Multiple tumors exceeding 5 cm in individual size are staged as T3a. T3b consists of one or more tumors that involve a major branch of the portal vein (right or left portal, does not include sectoral or segmental involvement) or hepatic vein (right, left or middle branches). T4 consists of tumors that perforate the visceral peritoneum or direct invade adjacent organs other than the gallbladder.

It is important to note that the AJCC TNM system is based on examination of the pathological resection specimen and is not equivalent to the OPTN staging system [70] that is used to qualify potential liver recipients in the US for additional MELD points while on the liver wait list. The OPTN system is based on the Milan criteria [71] and allows for additional MELD points in the case of a T2 tumor, which is defined as one tumor greater than 2 cm and no more than 5 cm in size, or 2–3 tumors at least 1 cm but no more than 3 cm in size.

A number of clinical or clinicopathologic staging systems have been proposed over the years for more precise prognostic subgrouping for HCC patients. The Barcelona Clinic Liver Cancer (BCLC) Staging System[72] is widely used in the West and uses the Child–Pugh score, tumor morphology, alpha-fetoprotein level, and portal vein thrombosis as independent predictive survival factors and to guide therapy [73]. Additional staging systems continue to be proposed as improvements over this approach [74]. An overview of the

current status of HCC staging is provided in a recent editorial by Sherman [75].

20.5.4 Microscopic Aspects

Hepatocellular carcinomas encompass 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 HCA [76, 77] 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. A number of specific histologic variants exist (fibrolamellar, clear cell, scirrhous, sarcomatoid, lymphoepithelioma-like and combined HCC-cholangiocarcinoma) and are considered separately (below).

The most common architectural pattern is an arrangement that caricatures the hepatic trabeculae (Fig. 20.6a). Normal trabeculae are 1–2 cells thick, evenly arranged, bordered by a well-developed reticulin network, and separated by sinusoids without prominent endothelial cells. In contrast, neoplastic pseudotrabeculae vary from 2 to over 20 cells in thickness, are irregularly arrayed, generally but not always have a reduced to absent reticulin framework, and are separated by a vascular network lined by endothelial cells and containing isolated arterial/arteriolar branches. The extent of microvessel density is considered to represent a negative prognostic indicator [78].

Other growth patterns of HCC are variations on this basic theme. A pseudoglandular (pseudoacinar) pattern arises from either dilatation of the bile canaliculi 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 with the appearance of "islands" of tumor cells, usually surrounded by a lining of endothelial cells [79]. A compact or solid pattern results when malignant cells closely appose one another, rendering sinusoidal or vascular spaces inapparent. It has been suggested that HCCs with a compact growth pattern have a better prognosis as compared with trabecular and acinar patterns [80]. Conversely, rare HCCs may display extensive peliotic change [81, 82], requiring distinction from peliosis hepatis or from normal hepatic response to vascular flow compromise. Another rare occurrence is the presence of isolated extracellular myxoid change in the absence of any evidence of biliary differentiation [37], a condition in which mixed HCC-cholangiocarcinoma should be carefully excluded.

Tumor cells typically 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 activities are increased in the tumor cell population. As HCC approaches moderately to poorly differentiated appearance there is a corresponding exaggeration of all of these features, with an increase in cell-to-cell heterogeneity and the emergence of giant or bizarre tumor cells in some cases (Fig. 20.6b). Different degrees of differentiation can be seen within a single tumor.

A variety of cytologic modifications may occur within a given case of HCC. In general these have no prognostic relevance, but can be useful clues for the diagnostic histopathologist. In some cases clear cells may predominate due to glycogen or lipid accumulation. Macrovesicular

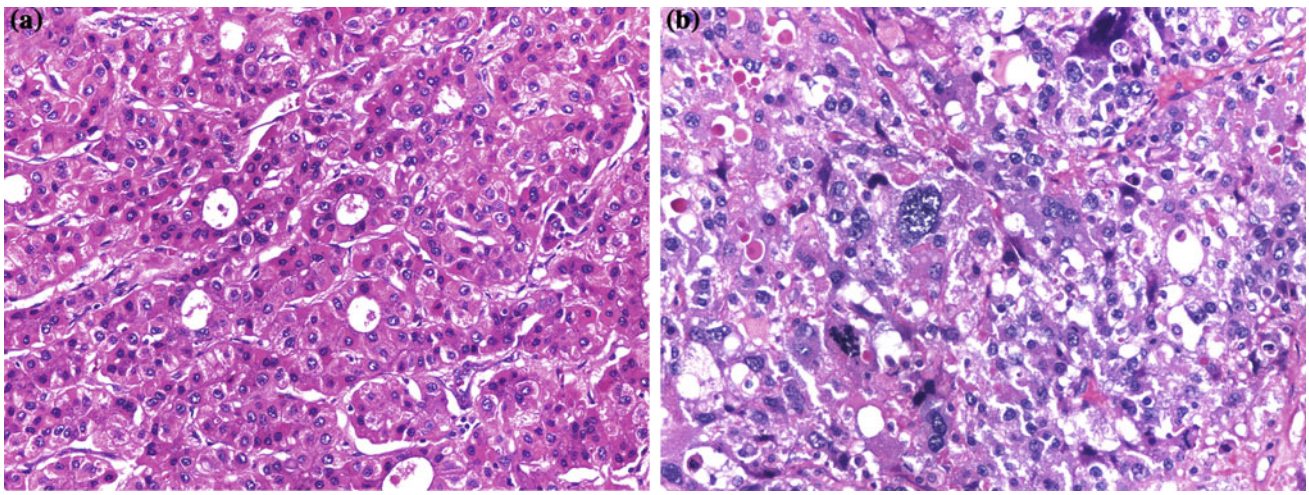

Fig. 20.6 Hepatocellular carcinoma. **a**. Well-differentiated HCC contains neoplastic cells forming small gland-like structures (pseudoacini). Nuclei show only slight variability. **b**. Poorly differentiated HCC. The tumor contains a bizarre giant cell (*center* of photomicrograph) with a large abnormal mitotic figure nearby. The architecture does not show the orderly arrangement of well-differentiated HCC

steatosis may be diffuse or focal and appears to be a more frequent finding in small HCC. Frankly steatohepatitic features such as ballooning degeneration have also been described in this context [83].

Bile pigment is noted in about 20 % of HCCs. Bile within neoplastic cells or bile canaliculi is an indicator of hepatocellular origin. A variety of other intracellular inclusions can be identified in individual cases. Dense eosinophilic globular bodies may be intra- or extracellular. These are usually PAS-positive and can contain various proteins including alpha-fetoprotein, alpha-1-antitrypsin, alpha-1 anti-chymotrypsin, 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 [84]. Pale bodies may simulate "ground glass" inclusions related to hepatitis B virus infection, but unlike true ground glass inclusions, they do not contain viral components [85, 86]. It has been suggested that proteins expressed in intracytoplasmic bodies might in some cases contribute to the malignant phenotype, since in one case p62, an IGF2 mRNA-binding protein associated with aggressive HCC phenotype, was identified as the major component of such inclusions [87, 88]. Mallory bodies occur in about 20 % of HCCs, regardless of underlying disease [89, 90]. 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 [91]. Rarely extramedullary hematopoiesis and granulomas can be detected. Kupffer cells are present but quantitatively reduced in HCCs, with more prominent decreases noted in larger and less well-differentiated tumors [92]. 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 [93].

The stroma of HCC is usually scanty. In some cases there can be a fibrous background and differentiation from other forms of adenocarcinoma or from mixed HCC-cholangiocarcinoma may become problematic and require immunohistochemical studies (below).

Distinct fibrous capsules frequently surround tumor nodules, 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 [94–96]. Well-developed encapsulation is more common in small HCCs. 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. Presence of a capsule may represent a manifestation of host defense that can interfere with the growth and invasiveness of HCC [94, 96]. It has been suggested that tumor infiltration of the peritumoral capsule or of the surrounding parenchyma correlates with a higher frequency of portal vein invasion and intrahepatic metastases [55].

Microscopic angiolymphatic invasion is not uncommon and should be specifically sought, as it represents a negative prognostic indicator. Less commonly, intrabiliary involvement on either a macroscopic or microscopic level may occur and has similar implications [97].

A four-tiered histologic grading system for HCC was originally put forth by Edmondson and Steiner [98]. Although pathologists universally claim to use this system, which relies on six characteristics stressing architectural and cytoplasmic features in addition to nuclear changes, in reality they use the Ishak modification [99] that relies exclusively on nuclear alterations. In this system, Grade I tumors contain cells with nuclei that closely simulate those of normal liver and diagnosis is dependent upon architectural features such as stromal or vascular invasion and/or aberrant trabeculae. Grade II cells have rounded to ovoid nuclei with a regular pattern of mild nuclear abnormalities including hyperchromatism and nuclear membrane irregularities. Grade 3 HCC has greater nuclear pleomorphism with the presence of angulated nuclei and more variability among cells, and Grade 4 HCC shows marked pleomorphism and hyperchromatism, usually with coexisting anaplastic giant cells. The AJCC currently provides the option of using a 2, 3, or 4 grade system and the pathologist should specify which system is being applied. Tumor grade correlates with the gross morphology, DNA content, proliferation markers, metastases, and AFP production and has been shown to represent an independent prognostic indicator for both survival and for tumor recurrence following resection [100, 101].

20.6 Immunocytochemical Aspects of Hepatocellular Carcinoma

Immunocytochemical studies are an important diagnostic adjunct and in some cases the detected antigens also have prognostic significance. Application of this technique for therapeutic guidance is not a part of routine evaluation since potential biomarkers are limited and have not been validated at this time. Each of these aspects will be considered.

Diagnostic immunocytochemical assessment of HCC may be necessary to answer one of several questions, depending upon the clinicopathologic circumstance: (A) is

the malignant tumor of hepatocellular versus metastatic origin? (B) if the tumor is of obvious hepatocellular origin, does it represent a carcinoma or other hepatocyte-derived lesion such as adenoma or high-grade dysplasia? (C) if the tumor is shown to be HCC, is there evidence for a mixed (cholangiocarcinoma) component?

To answer the first question requires establishing a hepatocellular phenotype. A number of antibodies are in use for this purpose, each with their own strengths and limitations. For this reason a panel approach is usually employed. Individual approaches vary but typically employ some combination of polyclonal carcinoembryonic antigen, CD10 (neprilysin), HepPar-1 and arginase-1. Antibodies to glypican-3 and alpha-fetoprotein also can be employed as first-line diagnostic reagents.

Immunostaining with the polyclonal anti-carcinoembryonic antigen (CEA) antibody highlights a bile canalicular pattern due to cross-reactivity with biliary glycoprotein in 60–90 % of HCC and was estimated in one series to be 79 % sensitive and 97 % specific for these tumors [102]. The antibody also detects normal bile canaliculi and cannot be used to distinguish benign from malignant status. Adenocarcinomas and cholangiocarcinomas will often show cytoplasmic staining, a pattern that is less common in HCC. Further, these other tumors can also react with the more specific monoclonal anti-CEA antibodies, which do not detect HCC. One caveat in our experience is that some commercially available monoclonal CEA antibodies do retain biliary glycoprotein cross-reactivity, and this possibility should be evaluated for the clone in use at a particular center. Ascertainment of what constitutes a "canalicular" pattern is also operator dependent. In well-differentiated tumors where adjunct studies are of marginal importance, a canalicular pattern is usually evident. In less well-differentiated tumors it may manifest as an incomplete membrane pattern with little resemblance to the well-ordered canaliculi of the non-neoplastic liver.

A canalicular pattern of staining in benign and malignant hepatocytes can also be demonstrated with antibody to CD10 (neprilysin) [103, 104]. In one study this antibody showed 68 % sensitivity and 100 % specificity for the differential diagnosis of HCC. The use of both polyclonal anti-CEA and anti-CD10 is recommended since an individual tumor may show positive uptake of only one of these two markers [105].

HepPar 1 [106] is a monoclonal antibody that detects the intramitochondrial urea cycle enzyme carbamoyl phosphate synthetase 1 [107]. It detects both benign and neoplastic liver cells and is not rigorously specific for the hepatocyte phenotype, as it may rarely be expressed in other cell and tumor types [108, 109]. However, one study showed HepPar1 to have 82 % sensitivity and 90 % specificity for the detection of HCCs [102]. When used as a part of a diagnostic panel its diagnostic accuracy is enhanced [102, 110–112]. HepPar-1 is more likely to be expressed in well differentiated as opposed to poorly differentiated tumors, a feature that limits its utility in problematic cases.

Arginase-1 is an intracytoplasmic enzyme that catalyzes the last step of the urea cycle, cleaving arginine to form ornithine and urea. Interestingly, immunocytochemistry shows the frequent presence of this enzyme in hepatocyte nuclei as well as cytoplasm. Arginase is reported to have a higher sensitivity and specificity than HepPar-1 for the diagnosis of HCC and the combined use of these reagents has been suggested [113, 114]. Arginase positivity does not appear to be dependent upon the differentiation grade of the tumor and has been suggested in a panel with glypican-3 to be of high sensitivity and specificity for distinguishing HCC from other carcinomas [115].

Glypicans are a family of six heparan sulfate proteoglycans that are mainly expressed in a stage- and tissue-specific manner during development [116]. One form, glypican-3, is highly transcribed in HCC [117] and can serve as a marker for this tumor. 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 [118] and in approximately 80 % of melanomas [119], all of these representing tumors that only rarely enter into the differential diagnosis. Glypican 3 is more sensitive in the detection of poorly differentiated as opposed to well-differentiated HCC [118]. It can rarely be detected in high-grade dysplastic nodules.

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 also extends to a lectin-reactive fraction of AFP (AFP-L3). Several studies have shown that serum AFP-L3-positive HCC patients have less well-differentiated tumors than do patients negative for this marker [120, 121]. AFP-L3 along with des-gamma-carboxyprothrombin (DCP) have recently been cleared by the FDA in the US as serum biomarkers for risk assessment of HCC development. Advantages and pitfalls of these assays have recently been reviewed by Li and Satomura [122]. In contrast to AFP, immunohistochemical staining for DCP has not been of high value in distinguishing benign from malignant hepatocyte nodules, as it was expressed in background liver [123].

A bewildering number of immunohistochemical markers have been proposed as being of prognostic significance in the analysis of HCC. In general, the studies are retrospective and examine a limited number of tissue markers, correlating them to disease-free or overall survival or the risk of tumor recurrence following therapy. However, independent validation by external groups, which represents one of the criteria of the European Association for the Study of the Liver (EASL) [124], has not been performed and no single immunohistochemical marker or group of markers has been widely accepted as a prognostic or therapeutic (theranostic) criterion in clinical practice. This task is further complicated by the fairly large number of potential driver and passenger genes in individual tumors, the role of adjacent liver tissue on tumor development and behavior, and the variety of conditions that may evolve to HCC with differing molecular alterations, impeding identification of potential oncogene addiction [125].

The underlying molecular biology of HCC is covered elsewhere in this book and is only briefly discussed here. Zucman Rossi et al. [46] have recently divided HCC into two broad molecular classes, namely a proliferation and a nonproliferation class. The proliferation class encompasses those tumors with enrichment of activated signals within pathways associated with cell proliferation, survival, histone modification and ubiquitination [126] and incorporates tumors with enhanced markers for progenitor cells. These tumors correlate with poor histologic differentiation status, vascular invasion, high AFP levels, are aggressive and associated with poor prognosis. The nonproliferation tumors show frequent presence of classical Wnt signaling, overexpression of EGF receptor and show evidence of immune signaling. These show better levels of histologic differentiation, lower AFP levels and better prognosis. Genomic profiling of either tumor [126] or tumor and adjacent tissue [127] supports these divisions. Nault et al. developed a 5-gene score to predict survival after liver resection, applicable to both proliferation and nonproliferation classes [128]. Molecular profiling has not yet been incorporated into clinical decision-making. However, it appears likely to do so, and the door remains open that progress made in molecular profiling will translate into a simple panel of correlative protein markers with the potential to refine current clinicopathologic patient stratification. One such example is the ability of immunocytochemistry to identify an active Wnt/βcatenin pathway by the nuclear translocation of βcatenin. A second potential role for immunocytochemistry may lie in identification of patient subgroups amenable to specific therapies, such as c-MET [129] or immune checkpoint inhibitors [130, 131], dependent upon the ability to demonstrate improved outcomes with such treatments.

20.6.1 Pathologic Variants of Hepatocellular Carcinoma

20.7 Fibrolamellar HCC

Fibrolamellar hepatocellular carcinoma (FL-HCC) is separable from ordinary HCC on the basis of macroscopic, histologic, ultrastructural and molecular features [132]. This distinctive variant of HCC occurs predominantly in young patients (90 % under 35 years of age) without cirrhosis [133]. El-Serag and Davila [134] 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 predominance in whites [134], with relative rarity in Asia [135], although it may be becoming more commonly recognized in that region [136].

The clinical presentation is typically vague, with components of abdominal pain, malaise, and weight loss [132]. Less common presentations include biliary obstruction, thrombophlebitis [137], or metastatic spread [138].

The tumors are solitary in 90 % of cases, on average from 9 to 14 cm at time of presentation [132]. This neoplasm is unique among hepatocellular tumors in that the majority arises in the smaller left hepatic lobe [104]. The fibrous component of FL-HCC often forms a central scar demonstrable by radiological techniques [139]. The fibrous component also provides increased firmness to the tumor in comparison to typical HCC and may undergo calcification. The pattern of scar formation may superficially mimic that seen in FNH.

Microscopically, there is usually a compact architectural growth pattern but trabecular or acinar patterns can be observed. The neoplastic cells are larger than normal hepatocytes (Fig. 20.7), polygonal in shape, and possess abundant granular, eosinophilic cytoplasm, a so-called "oncocytic" appearance, due to numerous swollen mitochondria [140]. Nuclei are vesicular, rounded, and have prominent nucleoli, the latter representing a characteristic diagnostic feature of this tumor. Mitoses are usually sparse; pleomorphism and multinucleation are infrequent. Tumor cells contain pale bodies reactive for fibrinogen and hyaline globular inclusion bodies may be present [141]. Intracellular bile production, fat, glycogen, copper and copper-associated protein can be detected [142]. 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 [143] or more typical HCC [144, 145]. Clear cell changes

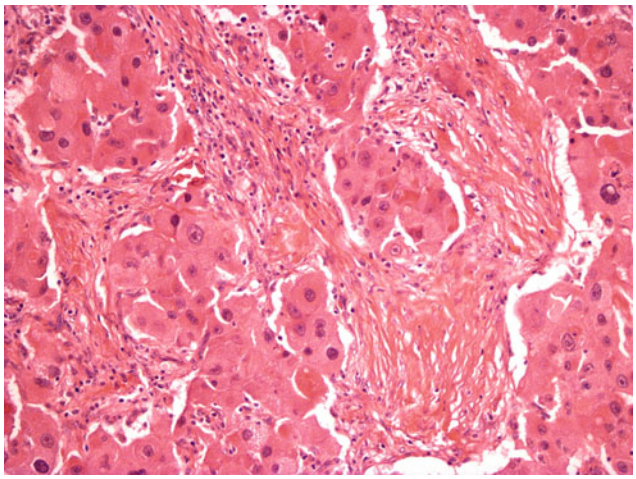

Fig. 20.7 Fibrolamellar HCC. Tumor cells contain a generous amount of cytoplasm and show variably prominent nucleoli. Intervening fibrosis has an orderly stacked or lamellar configuration (200×)

have been described in a case of otherwise typical fibrolamellar HCC [146]. Concomitant presence of conventional HCC and macroscopic vascular invasion have been associated with decreased survival in some series [147].

A prominent collagenous fibrous stroma that is arranged in thin parallel bands (lamellae) is a characteristic feature of fibrolamellar HCC but may be sparse or even absent in some tumors. Diagnosis is not absolutely dependent upon demonstration of the fibrous component. The collagen is predominantly composed of types I, III, and V [148]. 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 [149].

Tumor cells are positive for HepPar-1 [150], hepatocyte cytokeratins 8 and 18 and also contain biliary cytokeratins 7 and 19 [151] as well as CD68, typically associated with macrophages [152]. 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 [153], and prominent AFP positivity, particularly when combined with elevated serum levels, suggests that a search for areas of more typical HCC should be undertaken [154]. Glypican-3 immunopositivity was seen in 64 % of fibrolamellar HCC in one small series [155], and in some cases uptake was patchy.

The discovery of a novel fusion transcript, DNAJB1-PRKACA specifically in fibrolamellar HCC represents a major advance in molecular dissection of this tumor [156]. DNAJB1 is a member of the heat shock 40 protein family and PRKACA encodes cAMP-dependent protein kinase A catalytic subunit alpha. The fusion protein retains kinase activity and is thought to be expressed in 79–100 % of tumors [157, 158]. It is currently unknown whether this is a primary driver oncogene in this tumor, if it is secreted in the circulation or it represents a therapeutic target [159]. Diagnostic in situ hybridization has been performed to detect this RNA and successfully distinguished fibrolamellar from scirrhous HCC, a tumor that also can contain significant fibrosis [160].

Although no other recurrent genomic alterations have yet been identified, transcriptomic analysis of a fibrolamellar HCC cell line reveals a number of other genetic alterations that differ from those seen in typical HCC [158]. These studies also led the authors to conclude that fibrolamellar HCC represents a single disease and not a collection of different tumor types. Further, the upregulation of some neuroendocrine-associated genes was felt to represent an epiphenomenon, arguing against older interpretations that this tumor had a neuroendocrine origin.

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 [161–164]. Resectability is an important prognostic variable [165, 166], and Katzenstain et al. [167] 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. More recently, Darcy et al. [168] also found resectability to be an important factor for prolonged survival.

20.8 Clear Cell HCC

Clear cell HCC is comprised of malignant hepatocytes, the majority of which contain clear or empty-appearing cytoplasm reflecting accumulation of intracellular glycogen or lipid [169]. The tumor typically arises in a background of cirrhosis and has only rarely been reported in a non-cirrhotic setting [170]. Liu et al. [171] found an association of clear cell change with hepatitis C virus infection in an Asian series, and individual associations with non-alcoholic steatohepatitis [169], hypoglycemia and hypercholesterolemia [172] and tyrosinemia [173] have also been reported.

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 [174]. One complication arises from rare reports of concurrent clear cell primary hepatic and renal tumors [175], underscoring the necessity of phenotyping individual tumors when they occur in different sites. Adrenal tumors or adrenal rests may also cause difficulty as these may give a positive result with

HepPar-1 antibody, leading to an erroneous diagnosis of clear cell HCC [176]. Arginase-1 is negative in adrenal lesions, and use of this antibody in conjunction with adrenal markers such as CD56 or MART-1 circumvents this problem.

Several series [177, 178] found no difference in overall clinical behavior between clear cell and typical HCC. In contrast, Clayton et al. [179] found clear cell morphology to be associated with more favorable outcomes in hepatomas that diffusely infiltrated the liver ("cirrhotomimetic" pattern). Liu et al. [171] reported more frequent presence of a tumor capsule and lower rate of vascular invasion in clear cell tumors. Jeon et al. [180] report a remarkable case of an elderly male who experienced spontaneous regression of a large clear cell HCC with metastases.

One study [177] uncovered a clear cell HCC with microsatellite instability in contrast to other clear cell HCC in that series and concluded that clear cell HCC represents a heterogeneous entity. Orsatti et al. [181] also pointed to subtypes within this category. They showed that nondiploid clear cell HCC were more pleomorphic and had a higher mitotic rate than diploid tumors and suggested that such differences might account in part for differing opinions regarding the behavior of clear cell HCC.

20.9 Scirrhous (Sclerosing) HCC

Scirrhous HCC 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 [182]. 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 [183]. The margin is often ill-defined on CT scan [184]. Macroscopically, the mass is usually large, firm, and gray-white in color. The characteristic histological features of the sclerosing HCC are non-lamellar, extensive fibrosis (Fig. 20.8) that extends from the sinusoidal areas [185] and a pseudoacinar formation of the tumor cells. Tumor capsule formation is seen in about 30 % of cases or less [184, 186], and in one series vascular involvement was more common than in typical HCC [184]. Origin within a dysplastic nodule has been described [185].

The hepatocellular component of the tumor shows lower expression of HepPar-1 than ordinary HCC [232]. Krings et al. [187] found HepPar-1 uptake in only 26 % of scirrhous tumors in comparison to arginase-1 which detected 85 % of cases and glypican-3, positive in 79 %. The combined use of arginase-1 and glypican-3 allowed them to establish the correct diagnosis in 100 % of cases.

The sclerotic stroma, together with the occasional pseudoglandular pattern assumed by the tumor cells, may lead

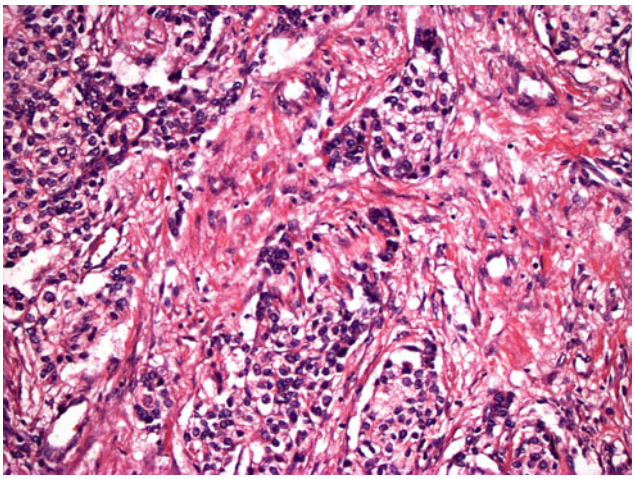

Fig. 20.8 Sclerosing HCC. The tumor cells do not contain the abundant cytoplasm of fibrolamellar HCC and do not typically exhibit prominent nucleoli. Fibrosis is well developed but does not show the exaggerated orderly pattern of fibrolamellar HCC (200×)

the diagnostic histopathologist to an incorrect diagnosis of cholangiocarcinoma. Markers typically associated with biliary phenotype such as cytokeratins 7 and 19 and epithelial cell adhesion molecule (EpCAM) may also be expressed [187]. Okamura et al. [188] 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 [188]. Presence of intracellular mucin would also favor cholangiocarcinoma (or metastatic adenocarcinoma).

An increase in fibrous stroma of HCC has been associated with an increased degree of stem cell phenotype [189] and expression of such markers as cytokeratin 19, EpCAM, or CD133 is associated with more aggressive biological behavior. In one study, scirrhous HCC was associated with a greater tendency for portal vein invasion as well as more frequent occurrence in non-cirrhotic liver compared to usual HCC [190]. Despite this, no difference in clinical outcome has been documented between scirrhous versus typical HCC [184, 190].

20.10 Combined Hepatocellular Cholangiocarcinoma

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 % [191]. The WHO recognizes this as a specific tumor type, whereas

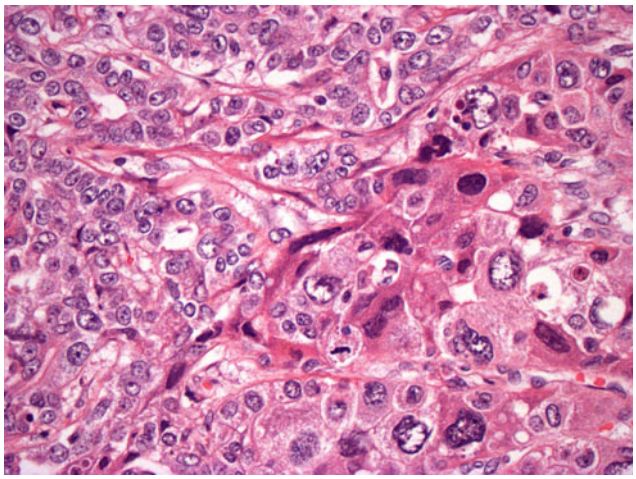

Fig. 20.9 Mixed HCC-cholangiocarcinoma. Compare the size of nuclei on the *left* side of the photomicrograph with those on the *right* to more easily appreciate the two different cell phenotypes present in this tumor. The more crowded cells on the *left* form glands and represent the cholangiocarcinoma component, whereas the cells with larger nuclei on the *right* appear to have a more solid arrangement and showed hepatocellular features by immunohistochemistry (400×)

the AJCC stages it as a cholangiocarcinoma. It has been associated with risk factors of HCC, such as HBV, HCV and alcohol use [192] and is considered to have a worse prognosis than standard HCC [193, 194] or even intrahepatic cholangiocarcinoma [195]. In a review of the SEER database, Garancini et al. [195A] found inferior survival for combined HCC-CC following liver transplantation as compared to HCC.

The tumor typically contains elements of both hepatocellular and cholangiocellular appearance (Fig. 20.9) and must be distinguished from rarely described synchronous and separable HCC and cholangiocarcinoma [197, 198]. It is thought that the divergent phenotypes of the combined tumor arise from a common stem or progenitor cell [199] and earlier studies did disclose shared features of both components. For example, Imai et al. [200] found similar p53 and RB-1 locus mutations in both hepatocellular and cholangiocellular components of mixed HCC/CC and a cell line derived from a human HCC/CC showed features of one or the other cell component dependent upon growth conditions [201]. Gil-Benso et al. [202] were also 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.

The tumor exhibits clinical behavior typical of either tumor type, such as vascular invasion as seen in HCC and lymph node metastases as occurs with cholangiocarcinoma. For that reason surgical intervention often employs lymph node dissection.

Pathologic diagnosis of the classic form of combined HCC-CC is dependent upon recognition of cellular and architectural features of both cell compartments. For this reason core biopsy is preferred over fine needle aspiration. The hepatocellular component is recognized by morphology and by features such as bile production. Immunohistochemical markers such as HepPar-1, arginase-1, or a canalicular pattern of staining with anti-polyclonal CEA or with CD10 are helpful in this regard. Alpha-fetoprotein is also positive to a lesser extent.

The cholangiocellular component is detectable with markers of biliary differentiation such as cytokeratins 7 or 19 and EpCAM (MOC31). Attention to the morphology of the cells being stained is important, since otherwise typical HCC may on occasion express either of these markers.

A second tumor category recognized by the WHO is that of combined HCC-CC with stem cell features. The putative stem cells are small undifferentiated intermediate or oval-like cells on the basis of both light and electron microscopy [203]. These cells also typically express positivity for cytokeratins 7 and 19 along with CD56, EpCAM, and c-kit, which are considered stem cell markers in this context. This category is further divided into several subtypes that are pathologically distinguishable but have no known clinical differences. The combined HCC-CC typical subtype contains nests of mature appearing neoplastic hepatocytes surrounded by smaller cells that represent the stem cells. A second group, the intermediate cell subtype, is comprised of nests or cords of cells intermediate between hepatocytes and cholangiocytes and showing markers of both cell types. C-kit expression is particularly frequent. The final subtype is the cholangiolocellular form, in which the cells form irregular tubules within a fibrous stroma, resembling cholangioles, and express stem cell markers.

Shafizadeh and Kakar [204] have proposed a spectrum of lesions that range from typical HCC to typical cholangiocarcinoma. The series in order includes HCC, CK19-positive HCC, scirrhous HCC, HCC with stem cell features, combined HCC-CC with stem cell features, classical type combined HCC-CC, (here we would add cholangiolocellular subtype) and finally cholangiocarcinoma. This is useful in conceptually organizing the various forms of these tumors but does not imply progression of one to another nor is it meant to suggest a parallel spectrum of molecular changes.

20.11 Sarcomatoid HCC

Sarcomatoid HCC is a rare variant of HCC that may contain spindle-shaped cells with features of any of a variety of sarcomas including fibrosarcoma, leiomyosarcoma, rhabdomyosarcoma, osteosarcoma, and others [205, 206]. The sarcomatoid component is considered to represent a form of tumor progression, "dedifferentiation" or epithelial-mesenchymal transformation 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 [206, 207]. Haratake et al. [207] suggest the keratin 8 positivity in the sarcomatoid element may be diagnostically helpful in distinguishing these tumors from true intrahepatic sarcomas.

Osteoclast-like cells may also be seen. These were previously thought to represent histiocytes on the basis of immunohistochemical studies [208] but more recently Dahm [209] showed that these cells co-expressed both macrophage markers (CD68) and HepPar-1, consistent with origin from the malignant hepatocytes.

Sarcomatoid change can also occur in mixed hepatocellular–cholangiocellular tumors [203, 210, 211] and the relationship between those tumors and sarcomatoid HCC is currently undefined. Sarcomatoid change has also been described following chemotherapy [212].

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.

20.12 Lymphoepithelial HCC

Lymphoepithelioma-like HCC is a rare variant characterized by significant numbers of tumor-infiltrating lymphocytes. It can occur in cirrhotic [213] or non-cirrhotic [214] liver and may be more common in females [214]. The inflammatory infiltrate appears to be T cell predominant, with both CD4 and CD8-positive cells demonstrable [214]. No association with Epstein–Barr virus has been demonstrated [213, 214]. Patient outcome compared to typical HCC is thought to be either favorable [213] or similar [214]. In contrast, a recent case report documented a patient who had rapid progression of a similar tumor [215]. Data are limited by the small numbers of studies, but it appears likely that the common denominator of large numbers of inflammatory cells may mask more complex or dissimilar host: tumor immune interactions in individual cases. Indeed the patient reported by Quist [215] failed to show significant caspase-3 uptake, indicative of apoptosis, in tumor cells despite the large number of infiltrating immune cells. Assessment of immune checkpoint inhibitors such as PD-L1 would be of interest in future cases.

20.13 Hepatoblastoma

20.13.1 Clinical Aspects

Hepatoblastoma is a rare tumor but represents the most common liver cancer in childhood. Risk factors include prematurity and low birth weight [216] and it can be associated with a number of inherited conditions including hemihypertrophy, Beckwith–Wiedemann syndrome, familial colonic polyposis, cardiac and renal malformations, Noonan syndrome, and glycogen storage disease type IA [217–222].

Clinically, an enlarging upper quadrant mass, vomiting, and/or fever are frequent presenting signs and symptoms. Serum alpha-fetoprotein is elevated in approximately 90 % of patients and an absence of this is associated with an aggressive course.

20.13.2 Macroscopic Aspects

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.

20.13.3 Microscopic Aspects and Ancillary Studies (Fig. 20.10)

Hepatoblastomas are thought to arise from a hepatocyte precursor cell and can have epithelial, mesenchymal and undifferentiated components. The Children's Oncology Group (COG) recently proposed an International Pediatric Liver Tumors Consensus Classification to standardize histopathologic diagnosis, particularly in the setting of international collaborative studies [223] (Table 20.2). This serves a different purpose than the Pretreatment Extent of

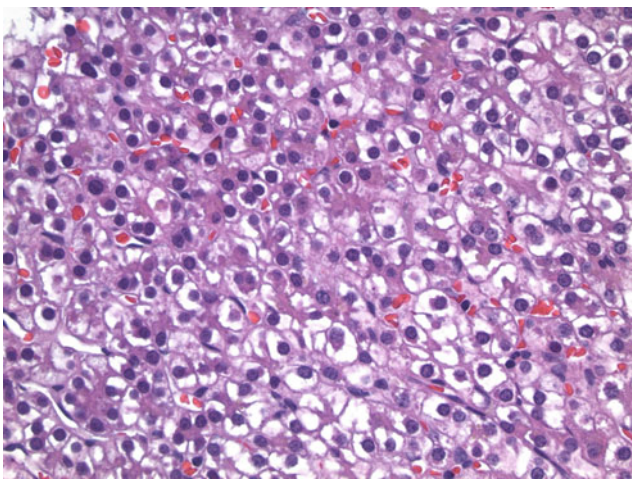

Fig. 20.10 Hepatoblastoma, fetal subtype. This well-differentiated tumor has no mitotic activity and consists of cells with abundant cytoplasm, round uniform central nuclei, and no apparent nucleoli (×400, image courtesy of Dr. S. Ranganathan, Children's Hospital Pittsburgh PA)

Table 20.2 Children's Oncology Group (COG) Classification of Hepatoblastomas [223]

1. Epithelial variants
a. Pure fetal with low mitotic activity
b. Fetal, mitotically active
c. Pleomorphic, poorly differentiated
d. Embryonal
e. Small cell undifferentiated
i. INI1-negative
ii. INI1-positive
f. Epithelial mixed (any/all above)
g. Cholangioblastic
h. Epithelial macrotrabecular pattern
2. Mixed epithelial and mesenchymal
a. Without teratoid features
b. With teratoid features

Disease (PRETEXT) which is used to stage the tumor prior to therapy and is predictive of event-free survival (reviewed in [224]).

Of the epithelial subtypes, the pure fetal form (Fig. 20.10) is a well-differentiated tumor comprised of medium sized bland malignant hepatocytes with varying amounts of intracytoplasmic lipid and glycogen. Mitotic activity is sparse and the tumors express alpha-fetoprotein. This is associated with a good prognosis and may be curable by surgery alone [225]; however, diagnosis requires extensive sampling of tumor which is typically not possible prior to chemotherapy.

Mitotically active fetal subtype (Fig. 20.11) is a second variant resembling and often coexisting with more

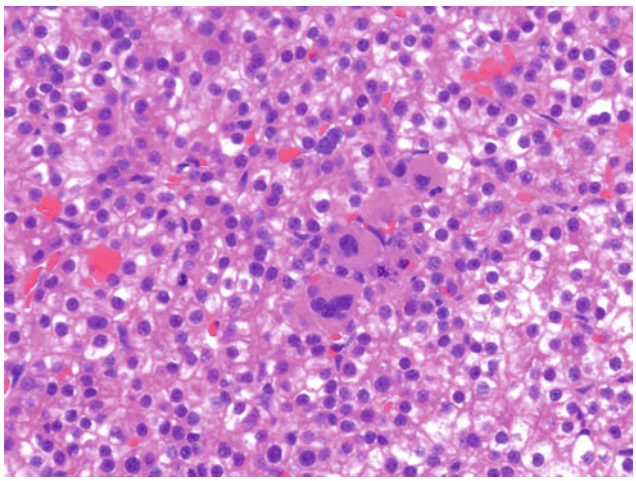

Fig. 20.11 Hepatoblastoma, fetal subtype, mitotically active. Nuclei are larger with more prominent nucleoli and more than 2 mitoses per 10 high power microscopic fields (×400, image courtesy of Dr. S. Ranganathan, Children's Hospital Pittsburgh PA)

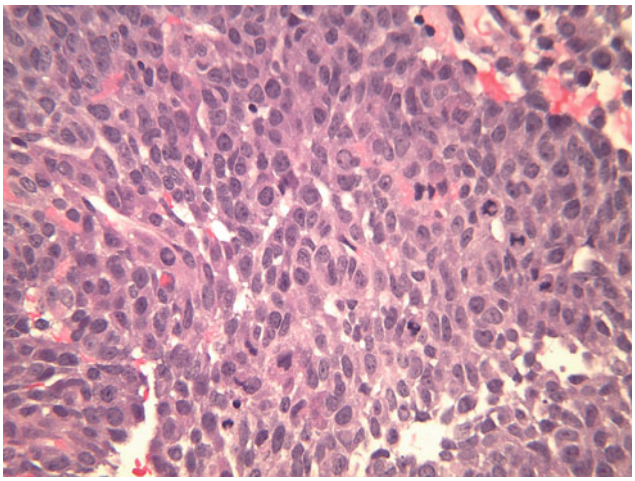

Fig. 20.12 Hepatoblastoma, embryonal variant. This tumor contains larger cells compared to fetal subtype and also shows an increased nuclear to cytoplasmic ratio with oval to angulated nuclei, variable nucleoli and frequent mitoses (×400, image courtesy of Dr. S. Ranganathan, Children's Hospital Pittsburgh PA)

well-differentiated fetal cells but exhibiting a higher nuclear to cytoplasmic ratio (hence the synonym "crowded fetal"), less cytoplasmic lipid or glycogen, more prominent nuclei, and mitotic activity in excess of 2 per 10,400× microscopic fields. These areas are also highlighted by coarser texture of glypican-3 stain relative to well-differentiated areas.

The pleomorphic epithelial variant remains recognizable as hepatocellular in origin but shows more nuclear variability with large nucleoli. Such changes are most commonly found after chemotherapy.

The embryonal variant (Fig. 20.12) usually does not represent a pure growth pattern but typically occurs in conjunction with a fetal subtype. These cells have little cytoplasm and do not contain lipid or glycogen. Nuclei are enlarged and hyperchromatic. The cells may arrange in solid aggregates or form glands.

Small cell undifferentiated (SCUD) form of hepatoblastoma (Fig. 20.13) is the second example of a histologic pattern with clinical import, in this case signifying an aggressive behavior. These cells have small bland round to oval nuclei with inapparent nucleoli and little cytoplasm. The may occur in sheets or be mixed with other epithelial types. Coexistence of keratin and vimentin positivity is consistent with their undifferentiated status and they do not express alpha-fetoprotein.

SCUD tumors have been further subclassified on the basis or absence of INI1 (SMARCB1). This is a core subunit of the SWI/SNF complex that participates in transcriptional regulation and chromatin remodeling [226]. It is thought to act as a tumor suppressor, and inactivation of INI1, which can easily be detected by immunohistochemistry, is associated with a wide variety of tumors that typically, but not

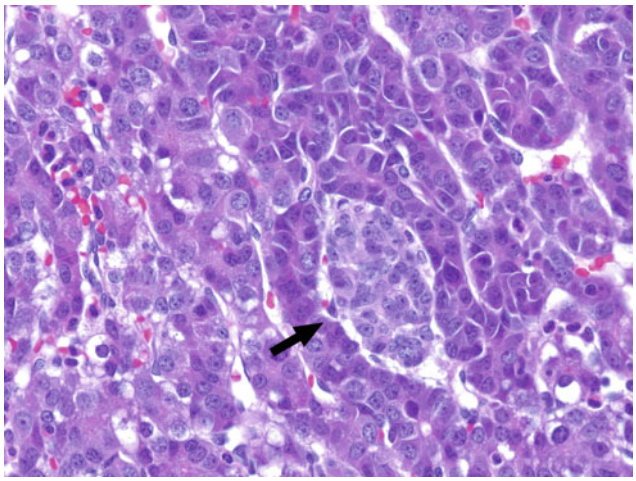

Fig. 20.13 Hepatoblastoma, embryonal with small cell undifferentiated component. The small cell undifferentiated component (*arrow*) consists of small uniform cells with inconspicuous nucleoli and scant mitoses. Surrounding embryonal cells are larger and show nucleoli and mitoses (×400, image courtesy of Dr. S. Ranganathan, Children's Hospital Pittsburgh PA)

invariably, contain a subset of large cells termed rhabdoid cells that have a superficial resemblance to rhabdomyoblasts [227]. SCUD tumors lacking INI1 have been suggested to have a better prognosis than INI1-positive tumors [228], and it has been suggested that these patients may be better served with therapy designed for malignant rhabdoid tumors [223].

Cholangioblastic tumors have a component of cells that resemble biliary epithelial cells and may form duct or ductular structures. These are identifiable with the biliary cytokeratins 7 and 19 and have not been shown to have independent clinically prognostic significance.

In contrast to the other subtypes, the macrotrabecular variant is not defined by cell type, but by an architectural arrangement in which thickened trabeculae at least 5 and up to greater than 20 cells wide is observed. This can occur with varying cell types, and the significance of this pattern, if any, is not yet clarified [223].

Slightly less than half of hepatoblastomas additionally have mesenchymal elements, most commonly osteoid and fibrous tissue. Teratoid features refer to the presence of additional complex elements of endoderm or neuroectodermal origin or to the presence of tissue components such as striated muscle. The full clinical significance of this remains to be defined.

The majority of hepatoblastomas have Wnt pathway signaling abnormalities, often due to β-catenin mutations [229] and demonstrable in tissue sections by nuclear translocation of β-catenin. Telomerase activation also occurs, and TERT can stimulate Wnt signaling independent of intrinsic Wnt pathway mutations. This is consistent with previous studies that found nuclear accumulation of β-catenin in the absence of beta catenin mutation [230, 231]. In a more recent study, Ueda et al. [232] found that patients with Wnt mutations were more responsive to therapy than those in whom Wnt activation was associated with TERT overexpression alone. MYC represents a separate Wnt target gene and has been associated with more aggressive behavior in an experimental setting [233, 234]. Incorporation of validated molecular assessments into the clinical setting is eagerly awaited.

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Protein Biomarkers in the Management of Hepatocellular Carcinoma: Novel Combinatory Approaches

21

Philip Johnson and Sarah Berhane

Contents

21.1 Introduction

It has been accurately stated that 'Tremendous efforts have been made over the past few decades to discover novel cancer biomarkers for use in clinical practice. However, a striking discrepancy exists between the effort directed toward biomarker discovery and the number of markers that make it into clinical practice' [19]. This is certainly true of the situation with regards hepatocellular carcinoma (HCC).

Reviews of biomarkers for HCC have conventionally focused on serum biomarkers and their role in diagnosis, particularly in the setting of surveillance. However, their role in assessment of prognosis and in monitoring response to therapy is also now starting to attract attention. The need for such biomarkers is well documented. Radiological/imaging approaches are increasingly recognised to have serious limitations. For example, ultrasound (US) examination which is widely used to 'screen' patients with chronic liver disease with a view to early diagnosis of HCC such that curative treatment can be applied has limited sensitivity. In the most detailed meta-analysis, the pooled figure for sensitivity was 63 % overall, rising to 70 % when screening was undertaken every six months as opposed to annually [43]. Current AASLD guidelines acknowledge that 'performance characteristics (of US) have not been well defined in cirrhotic livers' and that 'some patients, particularly the obese, are not good candidates (for surveillance) despite their risk' [7]. Issues also surround variability of equipment quality and US is very operator-dependent making good quality control difficult to achieve and document.

Herein, we describe some of the protein-based blood tests that have been proposed to fulfil the above roles, most likely in conjunction with US, and then describe how we have used the best described of these, combined where necessary, with other clinical features and liver function tests, to develop more accurate and entirely objective diagnostic and prognostic models. The overall performance of the biomarkers in question is reported as the area under the receiver operator curve (AUROC) supported by estimates of sensitivity and specificity.

21.2 Some Serum-Based HCC Biomarkers

21.2.1 Alpha-Fetoprotein (AFP)

Alpha-fetoprotein (AFP) is a foetal protein analogous to albumin in the adult. It almost disappears after birth as albumin secretion takes over and is only re-expressed in certain pathological conditions. One of these is HCC and

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since its discovery in the 1960s [1, 46] the extent to which it can be used as a diagnostic biomarker for HCC has been the source of great controversy [17, 32, 41]. The controversy surrounds the fact that about 30 % of HCC cases are 'AFP negative' and AFP levels may be raised in patients with chronic liver disease alone (i.e. not complicated by HCC). These observations clearly impact on the sensitivity of the test in a surveillance setting [36]. However, as a result of several reviews and meta-analyses the underlying figures are now fairly clear. The AUROC is between 0.8 and 0.85 (supported by two meta-analyses with pooled sensitivities of about 0.6) [48, 50]. Nonetheless the evidence that adding AFP to routine (US) examination increases the sensitivity of detection of early HCC is weak [43]. The figures in early disease (BCLC 0 and A) are, however, the really important ones and in the most detailed and careful analysis, Marrero et al. showed that these remained in this range for BCLC 0 and A, (AUROC 0.8 and sensitivity 0.65) [32]. There would thus appear to be a strong evidence-based case for AFP to act as a basis/backbone for surveillance and diagnosis, whilst recognising that some further source of sensitivity is likely to be required. This might take the form of new or other, non-related, biomarkers.

21.2.2 Des-gamma-Carboxy-Prothrombin (DCP)

Des-gamma-Carboxy-Prothrombin (DCP), also known as protein induced by vitamin K absence or antagonist-II (PIVKA-II) is an immature form of prothrombin [28, 45]. Elevated DCP values ($\geq$ 7.5 ng/ml) have been shown to be associated with a fivefold increased risk of developing HCC and on this basis DCP has received FDA approval. Based on a literature review of 20 publications, the overall sensitivity was 67 %, specificity 92 % and AUROC 0.89 [20] (Table 21.1). DCP and AFP are not closely correlated [6] and elevated levels of DCP occur in about 30 % of AFP negative cases making the case for the potential utility of a combination of the two [3, 10, 23]. Furthermore there have been intriguing suggestions that DCP levels can start to increase well before (up to 1 year) HCC is detected by conventional imaging techniques [3].

21.2.3 AFP-L3

AFP-L3 is a glycoprotein normally produced by foetal liver. There are three AFP glycoforms that can be separated on the basis of their lectin binding characteristics [39], most readily with *Lens culinaris* agglutinin (LCA). The structural variation of these glycoforms depends on the degree and siting of fucosylation of the N-acetylglucosamine-linked sugar chains. AFP-L3, the glycoform found in individuals with HCC, is characterised by binding to LCA with high affinity [27, 34, 39]. In adults, an increase in AFP-L3 appears more specific for HCC than total AFP [5, 22, 33, 39, 52]. It is usually presented as a percentage of the total AFP with a reference range of <10 %. Elevated levels have been shown to be associated with a sevenfold increased risk of developing HCC within the next 21 months. It is to be noted that the percentage is more significant than the absolute amount of AFP-L3, i.e. [AFP] $\times$ [AFP-L3].

21.2.4 Osteopontin

Osteopontin (OPN) is an integrin-binding glycophosphoprotein involved in many cellular functions, including invasion and metastasis. HCCs consistently express OPN at higher levels than normal tissue [40, 42]. It is found in the serum of healthy subjects and in patients with several different cancers including carcinomas of colon, pancreas and in multiple myeloma. In single centre analyses [8, 48], its performance was equal to or better than AFP among HCV positive patients and these findings have been confirmed in a recent meta-analysis [48] (Table 21.1).

Table 21.1 Test performance for some of the most intensively studied potential HCC biomarkers

	AUROC	Sensitivity	Specificity	Number of Publications assessed	Reference
AFP (1)		0.52		10	
AFP (2)	0.87	0.66	0.86	7	Wan et al. [48]
AFP-L3	0.76	0.48	0.92	12	Yi et al. [52]
DCP	0.89	0.67	0.92	20	Gao et al. [20]
Glypican-3	0.88	0.56	0.89	17	Liu et al. [30]
Glypican-3	0.82	0.53	0.77	12	Liu et al. [29]
Osteopontin	0.92	0.86	0.86	7	Wan et al. [48]
Golgi protein 7	0.86	0.77	0.91	11	Yang et al. [51]

21.2.5 Golgi Protein 73

This is a 73 Kd 400 amino acid transmembrane protein normally expressed on biliary epithelium but in pathological conditions it is expressed on the surface of hepatocytes, particularly malignant hepatocytes, and can be excreted into the circulation. Several studies suggest that it has the potential to be a sensitive serological marker for HCC [31, 37] with a performance similar to that of AFP [51].

21.2.6 Glypican-3

Glypican-3 (GPC3) is a proteoglycan attached to the cell surface by a glycosyl-phosphatidylinositol anchor [44] which is expressed by most HCCs but not in normal or cirrhotic liver. Immunostaining of GPC3 is widely used to confirm HCC diagnosis in diagnostic pathology [9]. Targeting of GPC3 might offer a new target for the treatment of HCC and clinical trials are ongoing [53]. GPC3 has been proposed as a serological marker [9, 16, 26] for HCC and in recent meta-analyses the pooled sensitivity was 56 % specificity 89 % and the AUROC 0.88 [29, 30].

21.3 Biomarker Combination

The general conclusion of the above, albeit selected studies, is that no individual marker, on its own, is likely to achieve the sensitivity and specificity required to have broad utility for diagnosis and surveillance, and we shall need either a new biomarker or a combination of existing biomarkers. Specifically, the evidence that adding AFP alone to routine (US) examination increases the sensitivity of detection of early HCC is weak [43]. Several groups have combined various biomarkers and, in general, shown improved performance [15, 21, 25, 49].

We have taken a purely practical approach in that any 'new' markers would require many years of study to become widely available and technically validated. In contrast, AFP has already undergone extensive studies such that there is a routinely available and well-validated assay. Further we believe that its performance in early disease is now convincingly demonstrated to the extent that it should represent the 'backbone' of any combinatorial serum-based model. Several groups have attempted to combine other markers, usually with AFP, and shown improved performance. Two other markers (AFP–L3 and DCP) are already commercially available on the same platform as AFP and both have been approved by the FDA for risk assessment of HCC. We now describe how we have combined these three biomarkers into statistical models for diagnosis, surveillance and prognosis. In current staging systems, continuous data (such as biomarkers) are regularly categorised for the purpose of simplicity. Such dichotomization of continuous variables is associated with loss of information, statistical power and introduction of bias in multiple regression procedures [14, 38]. It has also been shown that when a normally distributed variable is dichotomized at the median, it leads to loss of a third of the data [38]. In logistic regression analysis, categorization of continuous variables is associated with inflation of the type I error rate [2]. In the following examples we have used statistical approaches that maximises information extraction by using data in its continuous form.

21.3.1 THE GALAD Score

Based on a prospectively collected cohort of patients with HCC and a control group with chronic liver disease without HCC, we undertook a case-control study that aimed to develop a statistical model capable of predicting the probability of HCC in patients with chronic liver disease. Predictive variables associated with the presence of HCC were identified using a logistic regression with a parsimonious forward–backward stepwise approach and keeping variables significant at the 1 % level. The resulting model comprised **G**ender, **A**ge and the three biomarkers **A**FP, AFP-**L**3 and **D**CP hence, the acronym GALAD. The model was then validated on a further cohort prospectively accrued from the same institution and then on an external dataset from another UK institution [4, 25].

The GALAD mode uses the equation

$$Z = -10.08 + 0.09 \times \text{age} + 1.67 \times \text{sex} + 2.34 \log(\text{AFP}) + 0.04 \times \text{AFP} - \text{l3} + 1.33 \times \log(\text{DCP}) \quad (20.1)$$

where sex = 1 for males, 0 for females.

The linear predictor (Z) is used to estimate the probability of HCC in an individual patient (ranging from 0 to 1) using the following equation:

$$(\text{HCC}) = \exp(Z)/(1 + \exp(Z)) \quad (20.2)$$

The score can be calculated from Eq. 20.1 and then translated into a risk (from 0 to 1) from Eq. 20.2. This can be accomplished in a simple spreadsheet. Table 21.2 shows such a spreadsheet with several clinical scenarios.

The key performance characteristics for the model are shown in Table 21.3 and Fig. 21.1 (modified from Johnson et al. [25]. Our initial study showed that the model performed well in smaller tumours (<5 cm), Table 21.3 but subsequent studies have shown that the model is virtually uninfluenced by tumour size at least down to 2 cm. Preliminary analysis of our subsequent studies have suggested that the model performs equally well in other countries and is independent of

Table 21.2 Scenarios from a spreadsheet that automatically delivers the GALAD score ('*Z*') and the probability of HCC according to the biomarkers that are fed in

ID	Gender (0 = female, 1 = male)	Age (years)	AFP-L3 (%)	AFP (ng/ml)	DCP (ng/ml)	*Z*	HCC probability
Reference row	1	55	9	500	5	4.145	0.984
A	1	65	9	55	24	3.708	0.976
B	1	50	50	20	7	2.258	0.905
C	1	55	4	1	3	−2.665	0.065
D	1	55	1	5	2	−1.384	0.200
E	0	70	4	65	3	1.257	0.778
F	0	45	1	2	1	−5.286	0.005

Table 21.3 Performance of model on early and late stage patients (reproduced from [25])

			Max. sensitivity	Max. specificity	Max. both
			Cut-off = −1.36	Cut-off = 0.88	Cut-off = −0.63
Staging system/ Treatment type	Criteria for early or late disease	Number of Patients		Sensitivity	
BCLC					
Early	0 and A	42	93	55	86
Late	B, C and D	327	96	83	94
Tumour size					
Early	≤ 5 cm	169	92	67	88
Late	>5 cm	166	99	92	98
Treatment intent					
Early	Curative	61	85	56	75
Late	Palliative	252	98	86	98

Abbreviations: *BCLC* Barcelona Clinic Liver Cancer Classification

etiology or tumour size. Furthermore, the model, is not influenced in cases of chronic viral hepatitis C, by whether or not sustained virological response (SVR) has been achieved or in the case of chronic hepatitis B virus infection, whether or not patients are treated with antiviral agents.

21.3.2 The BALAD Score

The BALAD score was originally developed by Toyoda et al. [47] to assess prognosis in HCC. It has been externally validated by Chan et al. [11]; the acronym refers to **B**ilirubin, **A**lbumin, AFP-**L**3, **A**FP and **D**CP. These authors developed the model empirically based on the application of conventional cut-off points. When the same data set was assessed using rigorous statistical methodology (specifically using the data in a continuous format) the same individual parameters were identified. The performance of the model [18] that was built upon these variables (BALAD-2) was very similar, paying testament to the power of clinical intuition/experience. The model can be used to place HCC patients in one of four classes that define prognosis (Fig. 21.2).

With appropriate recalibration the model proved applicable to the UK population (figures—as above). Subsequently, we validated the model in a larger number of patients and in other countries where the spectrum of etiology is dissimilar from those in which the model was built and validated. We have also shown that the model offers clear discrimination at different disease stages from early to advanced cases. The accuracy of the BALAD score is plausibly explained if the 'LAD' reflects prognosis attributable to tumour related factors and the 'BA' reflects the prognosis attributable to the associated chronic liver disease (Fig. 21.3). This contention has been substantiated in a recent study that established a new score for liver disease amongst patients with HCC.

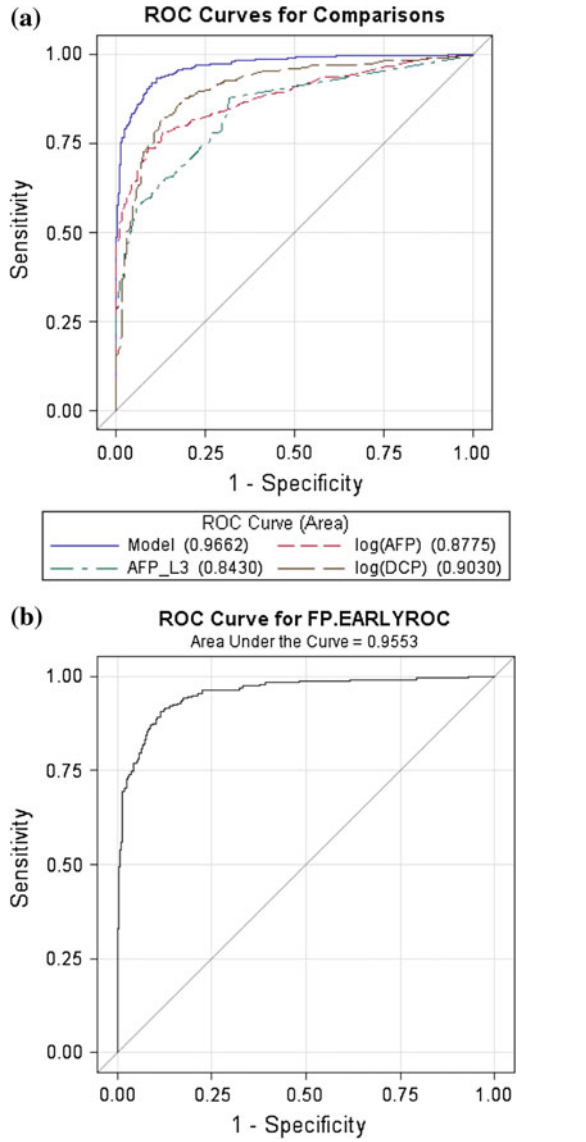

Fig. 21.1 Performance of the GALAD model compared to that of **a** individual contributing biomarkers and confined to **b** patients with 'small' tumours (defined as maximum diameter <5 cm). Reproduced from [25]

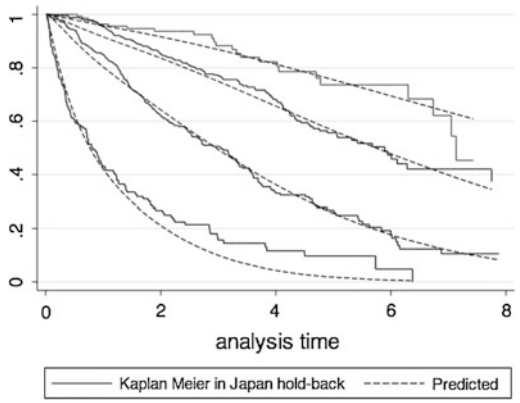

Fig. 21.2 Kaplan Meier curves depicting actual (*solid line*) and predicted (*dashed line*) survival using BALAD-2 model. Figure reproduced from [18]

21.3.3 The ALBI Score

As noted elsewhere in this book, most patients who develop HCC do so, on the basis of underlying chronic liver disease that has often progressed to the stage of cirrhosis by the time HCC presents. This is of crucial importance in the management of HCC since it appears to be an independent factor influencing survival both directly and indirectly by limiting some of the potentially curative treatments such as surgical resection.

Conventionally the Child-Pugh score/grade (CPS) has been used to assess liver function despite the fact that it was not designed for this application [12, 35]. Furthermore, it relies on assessment and quantification of ascites and encephalopathy that are both highly subjective, a concern which is amplified by the numerous (>30) versions of the CPS which are described in the literature. Some of these offer different scoring for the same degree of dysfunction (for example 'moderate' ascites may score I point in some versions and 2 points in others). Such inherent inconsistencies of the CPS are important since the difference of one point can move a patient from one class to another and may impact on their subsequent treatment.

For all these reasons we undertook a detailed analysis of the factors influencing survival in HCC and identified those that were independently associated with measures of liver (dys) function and survival. In the event the two major liver-related factors were serum albumin (AL) and bilirubin (BI), hence ALBI. This result was plausible as these two factors have been identified as those most closely associated with prognosis in cirrhosis [13].

The model is easy to apply using a heat map [24] and entirely objective. It is important to recognise that the model is not claimed to be a prognostic model for HCC; it is for assessment of 'liver function'. Further it is not claimed to be superior to the CPS but rather equivalent. The 'benefit' is reflected in several aspects:

- The CPS relies on five variables, whereas the ALBI score gives at least equivalent discriminations but only relies on two of these. It also suffers from all the limitations referred to previously of analyses that treat continuous variables in a categorical manner.
- The ALBI score shows that the two highly subjective variables of ascites and encephalopathy are redundant.
- The ALBI score has been extensively validated and performs well irrespective of the stage of disease. By convention CPS is only applied to patients with cirrhosis.

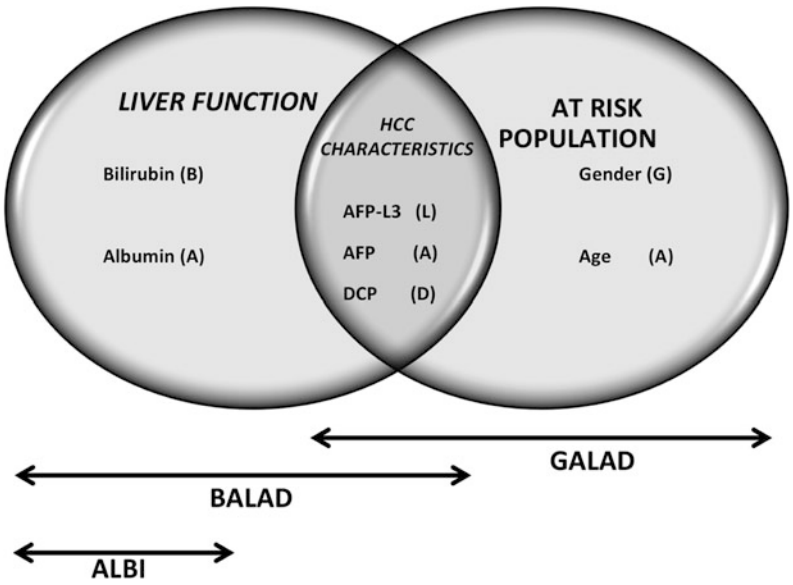

Fig. 21.3 Interrelationship of the three models described

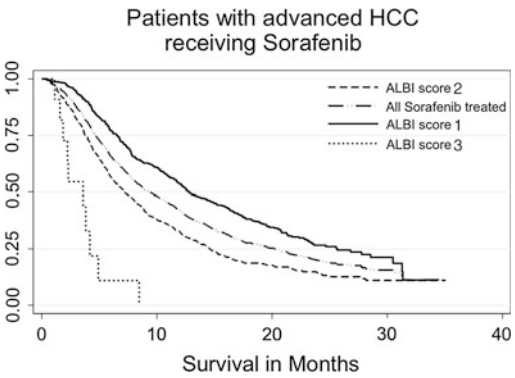

Fig. 21.4 Kaplan Meier curves showing the performance of the ALBI model in C-P 'A' patients undergoing Sorafenib treatment as part of a clinical trial. Reproduced from [24]

Table 21.4 Performance of GALAD model on early and late stage patients (reproduced from [25])

ALBI score	*N*	Median survival, months (95 % Conf. interval)
1	475	12.7 (11.7–14.9)
2	542	7.2 (6.4–8.2)
3	11	3.6 (1.6–4.9)
All Sorafenib treated	1028	9.3 (8.5–10.5)

- The ALBI score correlates closely with indocyanine green clearance, the closest test we have to a 'gold standard' for liver function.
- ALBI permits differentiation within individual CPS stages [24] (Fig. 21.4).
- ALBI gives much 'finer' reporting of liver function such that it can be monitored in relation to different treatments.

21.4 Conclusions

The models described offer simple, quantitative and objective approaches to the diagnosis and assessment of prognosis in HCC that are evidence-based. The utilisation of biomarker data in its continuous form rather than categorisation/di or tri-chotomisation according to cut-off points clearly results in a richer extraction of information that leads to improvement in test performance (Table 21.4).

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Surveillance for Hepatocellular Carcinoma

22

Cristina Della Corte and Massimo Colombo

Contents

22.1 Introduction

The global incidence and mortality rates of hepatocellular carcinoma (HCC) overlap worldwide, a fact that clearly indicates that majority of patients are identified with an advanced cancer that almost invariably prevents potentially curative treatments, thereby resulting in an average survival of 1 year from diagnosis [1–4]. The only hope for a cure, in fact, rests on early diagnosis as it may be obtained through surveillance of patients at risk, an end-point that unfortunately is achieved in a minority of patients, most clustering in the developed world [5]. Yet, population-based studies indicate that even in economically developed regions only a minority of patients with an HCC will ultimately undergo regular screening and curative treatments, despite most doctors and patients are fully aware of the benefits of screening for such a potentially lethal disease as HCC [6, 7]. This clearly underlines the existence of barriers to screening like limited or outdated knowledge, lack of financial incentives, limited access to appropriate testing and treatment, which altogether work against screening effectiveness. This is no surprise, since surveillance involves more than simply a screening test, whereas it is framed in a program where tests, recall policies, and quality control procedures are standardized, with significant economic consequences [8].

22.2 Target Population

HCC is unique in that it develops in the context of well-known and readily identifiable environmental risk factors. Indeed, majority of HCCs occur in patients with chronic liver disease including cirrhosis caused by chronic infection with the hepatitis B (HBV) and C (HCV) viruses and excess of alcohol intake [9, 10]. More recently, metabolic diseases related to insulin resistance, including diabetes and obesity, have been recognized to be causally related to HCC as well, in most patients bridging HCC to the histopathological diagnosis of non-alcoholic steatohepatitis

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Table 22.1 Groups for whom HCC surveillance is recommended or in whom the risk of HCC is increased, but surveillance benefit is incertain [8]

	Threshold incidence for efficacy of surveillance (>0.25 LYG) (%/year)	Incidence of HCC
Surveillance recommended		
Asian male hepatitis B carriers > 40 years	0.2	0.4–0.6 %/year
Asian female hepatitis B carriers > 50 years	0.2	0.3–0.6 %/year
Hep B carriers with family history of HCC	0.2	Higher incidence than without family history
African/North american blacks with hep B	0.2	HCC occurs at a younger age
Cirrhotic hep B carriers	0.2–1.5	3–8 %
Hep C cirrhosis	1.5	3–5 %
Stage 4 primary biliary cirrhosis	1.5	3–5 %
Genetic hemocromatosis and cirrhosis	1.5	Unknown, but probably > 1.5 %/year
Alpha 1 antitrypsin deficiency and cirrhosis	1.5	Unknown, but probably > 1.5 %/year
Other cirrhosis	1.5	Unknown
Surveillance benefit uncertain		
Hep B carriers younger than 40 (males) or 50 (females)	0.2	0.2 %/year
Hep C and stage fibrosis 3	1.5	<1.5 %/year
Non-cirrhotic NAFLD	1.5	<1.5 %/year

(NASH) [11–14]. Since the decision to enter a patient into a surveillance program is driven by the level of risk for HCC (Table 22.1), the incidence of HCC is generally taken as a starting point to select the target population to be screened. In the absence of experimental data to indicate what level of risk or what incidence of HCC should trigger surveillance [15], decision analysis/cost models have extensively been used to identify the incidence cut-off of HCC at which surveillance is worth [16]. While any intervention is considered effective whenever it provides an increase in longevity of about 100 days, the same intervention is considered cost-effective if achieved at a cost of less than US $50,000/year of life gained [16]. In Caucasian patients with Child–Pugh A cirrhosis, a 1.5 %/year incidence of HCC has been associated to about 3 month increase in longevity in a patient population lacking access to liver transplantation [17], whereas in a similar analysis including liver transplantation in a population of hepatitis C patients with cirrhosis, surveillance with either computed tomography (CT) scan alone or CT scan plus ultrasound (US) became cost-effective at HCC incidence rates of more than 1.4 %. Mitigating however the clinical impact of these models where the performance characteristics of CT scan being evaluated in diagnostic studies, not in the context of screening programs [18]. While biannual surveillance combining alpha-fetoprotein (AFP) with US was deemed cost-effective regardless of HCC incidence, by others [19]. Therefore, with all the caveats of data obtained through modeling, it seems reasonable to offer semiannual surveillance to patients with cirrhosis of varying etiology whenever the risk of HCC is 1.5 %/year or greater [8, 20].

Owing to the fact that cost-effectiveness analyses were restricted to cirrhotic populations, there are only sparse data on whether surveillance is worth in cirrhosis-free patients with chronic viral hepatitis. To our knowledge there is one cost-effectiveness analysis of surveillance for hepatitis B carriers using US and AFP levels only, which suggested cost-effectiveness of surveillance every 6–12 months in populations with an incidence of HCC exceeding 0.2 %/year (J. Collier and M. Sherman, unpublished observations). Currently, the American (AASLD) and the European (EASL) Associations for the Study of the Liver recommend surveillance for patients with cirrhosis of any etiology and for selected hepatitis B carriers using abdominal US at 6-month intervals, whereas the use of serum AFP as a surveillance test is discouraged [8, 20]. It should be acknowledged, however, that real-life studies of surveillance of patients with compensated cirrhosis of any etiology have highlighted high rates of non-HCC-related mortality that fuel the argument of cost-effectiveness of screening for liver cancer in the cirrhotic population [21]. Arguments are likely to be boosted by EASL recommendation of screening also hepatitis C patients with bridging fibrosis in addition to those with histological or clinical evidence of cirrhosis, since the transition from advanced fibrosis to cirrhosis could not be accurately documented in all patients [20]. The Asian Pacific Association for the Study of the Liver (APASL) endorses surveillance for cirrhotic patients with HBV and HCV maintaining the combination of US and AFP every 6 months [22]. Finally, surveillance for HCC is not endorsed at all by the National Cancer Institute which in fact questions the robustness and limited generalizability of data obtained so

far to elaborate the current guidelines, arguing on the lack of evidence that HCC mortality is decreased by surveillance [23]. This position is shared by others in the USA [24].

22.2.1 HBV Carriers as Target

The annual incidence of HCC in patients with chronic hepatitis B ranges from 2 to 5 %, in strict correlation with the histological stage of the underlying liver disease [25]. In Europe, HBV-related HCC is associated with cirrhosis in the majority of the patients [26, 27], whereas this is not true in Asia and Africa where the tumor is common also among carriers with mild hepatic fibrosis, likely as a consequence of long-standing infection that is often acquired perinatally [28–30]. Recently, it has been clearly demonstrated that also Asian carriers with inactive hepatitis, i.e., those with persistently normal ALT and serum HBV DNA < 2000 IU/ml develop HCC, yet at lower rates compared to patients with elevated viremia [26, 27, 31, 32]. In HBV patients, HCC risk may be modulated by additional risk factors like age, co-infection with hepatitis C or HIV, alcohol abuse, or co-presence of metabolic liver diseases. According to AASLD and EASL, surveillance is recommended independently on the level of fibrosis and ethnicity, to all adults with active hepatitis B. The REVEAL study and other population studies have clearly shown the existence of a direct relationship between the risk of developing HCC and viral load, even when this predictor was measured years before tumor diagnosis [32, 33]. This was clearly anticipated by prospective studies of cohorts of carriers from Europe and Asia in which the presence of serum HBeAg and high levels of HBV DNA were found to independently predict the subsequent development of cirrhosis and HCC [32, 34–37]. The fact that most carriers in Far East likely acquired HBV infection perinatally and had a mean age at enrollment of 40 years, drove the attention towards high levels of HBV replication persisting for more than 4 decades as a predictor of increased HCC risk [38, 39]. An intriguing finding of some studies, however, was the persistence of HCC risk in aged patients following HBsAg seroconversion, supporting both the carcinogenic role of occult infection with HBV and the need for continued surveillance of these patients [40, 41]. This is not the rule in Caucasian patients who were successfully treated with antivirals, in whom a decline of HCC risk following HBsAg seroconversion was annotated, likely reflecting differences in HBV epidemiology and modality of infection between Asian and Caucasian populations [42–45] (by courtesy of WR Kim, Stanford University). The fact that the yearly risk of HCC in male carriers in Southeast Asia starts to exceed 0.2 % at the age of 40 years, irrespectively of liver disease activity (J. Collier and M. Sherman, unpublished observations), led AASLD to endorse screening of Asian men from the age of 40 onwards. On the other hand, surveillance is recommended for 50 year-old Asian women due to their lower incidence of HCC compared to men. In patients with a family history of HCC, surveillance should be offered at a younger age, although the preferred age cut-off is not established [28, 46]. Since in African carriers HCC develops at a younger age compared to Caucasians, surveillance in these populations is deemed necessary at younger age than elsewhere. This is not the case for blacks born outside Africa [29, 30].

The HBV genotype has been implicated as a driver of cancer risk, probably as a consequence of genotype-related differences in duration and severity of HBV-related hepatic inflammation over time. Studies from Asia involved the genotype B in anticipated HBeAg seroconversion, higher rates of sustained remission after HBeAg seroconversion, less active hepatic necroinflammation, slower progression to cirrhosis, and lower rates of HCC development compared to genotype C of HBV [47–52]. Growing evidence suggests that genotype A infections have a generally more favorable outcome than genotype D infections in the West [53, 54]. With all the caveats due to a bias of patient selection, studies in Asia and West recognized that long-term administration of nucleo(s)tide analogs prevents the onset of HCC in patients with chronic hepatitis B, not in cirrhosis where the rates of cancer are lower than in untreated patients [55–57]. All liver societies, therefore, recommend continuing surveillance in treated patients including cirrhosis achieving HBsAg seroclearance.

22.2.2 HCV Carriers as Target

AASLD, EASL, and APASL, all endorse screening for patients with hepatitis C-associated cirrhosis. While the incidence of HCV-related tumors is declining in southern Europe and Japan, HCC is on the rise in other geographical areas including United States and northern Europe, all these changes being related to a modification of population exposure to viral hepatitis and alcohol [55]. Several retrospective and prospective studies indicate a wide range of HCC incidence in patients with hepatitis C-related cirrhosis which in fact spans from 2 to 8 % [58–60]. Conversely, there is a single prospective population-based study evaluating the risk of HCC in patients with chronic hepatitis C [61]. That study carried in 12.008 serum anti-HCV-positive men, demonstrated a 20-fold increased risk of HCC compared to anti-HCV negative subjects, without showing any correlation with presence or absence of cirrhosis. The HALT C study, originally designed to test the efficacy of chronic interferon dosing in patients with a previous failure to antiviral therapy, did confirm the occurrence of HCC in non-cirrhotic patients with chronic hepatitis C (5-year risk of 4.8 %), providing also the opportunity of constructing a risk score for HCC by combining factors like older age, African-American

ethnicity, lower platelet count, high alkaline phosphatase activity, and presence of esophageal varices [62].

Studies carried out in the West and Asia demonstrated that the risk of HCC is attenuated in cirrhotic patients with a response to interferon-based regimens [63]. However, since viral eradication does not completely eliminate the risk of HCC in older patients and those with advanced fibrosis, surveillance is worth to be continued in patients with cirrhosis following interferon related clearance of HCV-RNA [8, 20]. Liver cancer has been reported in fact to occur years after treatment completion, in some studies at a rate between 0.66 and 1.24 per 100 person years [46, 64], in others at rates between 0.6 and 2.5 % per year [65, 66]. In a French single center cohort study [55] and in many retrospective *studies* [64, 65] in cirrhosis, liver-related complications, including HCC occurred even after achievement of an SVR, reflecting the carcinogenic effect of the extensive architectural changes of the cirrhotic liver that may persist following an SVR. Another prospective Japanese study confirmed these results [67]. The similar cumulative incidence rates of HCC in patients with bridging fibrosis and those with cirrhosis highlight the need to treat HCV patients before the stage of bridging fibrosis. In one study [68] HCC after SVR was seen in patients with persistence of cirrhosis, not in those in whom cirrhosis reverted following antiviral therapy. In a retrospective study of more than 800 SVR patients in Japan occurrence of HCC was associated to a more severe liver disease score composed by age, platelet count, liver fibrosis, and AFP [69]. As the risk of HCC is high in HCV-cirrhotics who fail to achieve an SVR to interferon-based therapy [63, 64, 70–72], alternative treatment regimens have been explored. The administration of a long course of low dose of PegIFNα2a provided no benefit to the overall population, even though a small benefit in terms of HCC reduction was seen in patients classified as cirrhotics at baseline compared to those with advanced fibrosis (cumulative HCC incidence: 6.8 % vs. 15.5 %, $p = 0.01$) [73]. However, a similar study with PegIFNα2b failed to demonstrate any HCC prevention in both patients with cirrhosis and those with advanced liver fibrosis [74].

22.2.3 HIV and Viral Hepatitis as Target

In HIV infected patients liver-related morbidity and mortality significantly increased during the HAART era as a consequence of an important reduction in HIV-related complications, making co-infection with HBV (6–14 %) and HCV (25–30 %), to emerge as hepatotoxic factors in addition to excessive alcohol consumption, non-alcoholic fatty liver disease, and drug-induced liver injury [75].

While the MORTAVIC study in 2001 indicated HCC to be responsible for 25 % of all liver deaths, in the HAART era studies suggest that HCC developing in co-infected patients is more aggressive, presents at an earlier age and is less frequently curable than HCC in HCV mono-infected patients [76, 77]. If confirmed, these observations might lead to shortening of the interval between US examinations or extending the surveillance programs to all HIV co-infected patients, regardless of liver disease stage. Currently, the criteria for entering HIV co-infected patients into programs for HCC screening are the same as for mono-infected patients, i.e., based on the stage of liver disease as previously discussed.

22.2.4 Cirrhosis of Non-viral Etiology as Target

The incidence of HCC in cirrhosis caused by diseases other than viral hepatitis is—with some exceptions—poorly defined. Chronic consumption of more than 80 g of ethanol per day for more than 10 years increases the risk for HCC by approximately fivefold, not to forget, however, that alcohol consumption of 10 g/day in women is associated with a 24 % increase of HCC risk [78]. Alcohol abuse in patients with chronic hepatitis C doubles the risk for HCC as compared with the risk in teetotaler carriers of HCV, since there may be a synergism between alcohol and hepatitis C in anticipating HCC onset or causing more severe histological pattern of tumor [79]. In a HCC cohort in Austria, alcoholic liver disease was the likely cause of HCC in 35 % of subjects [10], whereas in the United States, the hospitalization rate for HCC-related to alcoholic cirrhosis is 8–9/100,000/year compared to about 7/100,000/year for hepatitis C [11]. Altogether, this data indicates patients with alcoholic liver disease to warrant surveillance for HCC, as recommended by AASLD [8]. However, this may not be the case in other geographical areas like northern European countries where mortality in alcoholics is mainly related to acute on chronic liver failure rather than to HCC, a fact that discourages surveillance of cirrhotic alcoholics in terms of cost-effectiveness [80].

In the last two decades NASH has been increasingly recognized as a cause of cirrhosis and HCC, whereby many patients can progress to liver cancer without histological evidence of advanced fibrosis or cirrhosis [81, 82]. A recent analysis of patients referred for liver transplant evaluation at Clifford Hospital demonstrated a yearly cumulative incidence of HCC in 2.6 % of patients with NASH compared to 4.0 % of those with HCV over a median follow-up time of 3.2 years [83]. Older age at the time of cirrhosis diagnosis and any alcohol consumption were independently associated with the development of HCC in NASH-cirrhosis population, suggesting that alcohol intake, even in socially accepted amounts, may potentially increase the risk of HCC development both in NASH- and HCV-cirrhotic patients.

Findings from a SEER based reanalysis, suggested that diabetes is an independent risk factor for HCC being associated with a two- to threefold increase in the risk of HCC, regardless of the presence of other major HCC risk factors [14]. In parallel, a case control study in Italy provided further evidence that obesity and diabetes are either jointly or independently associated with an increased risk of HCC, likely accounting for a relevant number of HCC cases among subjects lacking markers of HBV/HCV infection [84]. Several large-scale epidemiological studies have associated the increasingly overweight prevalence and obesity among the general population with a higher risk of HCC [85, 86]. In a cohort of 900,000 American adults, the risk of dying from liver cancer was 4.5 times higher in men with a body mass index of 35 kg/m^2 or above compared to the reference group with a normal body mass index (18.5–24.9 kg/m^2) [85]. A meta-analysis of case control and cohort studies concluded that the relative risk of liver cancer was 1.17 for overweight subjects and 1.89 for the obese patients [87]. Major systemic and liver-specific molecular mechanisms like insulin resistance, hyperinsulinemia, increased tumor necrosis factor signaling pathways, and lipotoxicity all together drive the development of HCC in this set of metabolic diseases. As a matter of fact, both metformin and PPAR (Peroxisome proliferator-activated receptor)-gamma agonists that are active components of oral treatment of diabetes, have been associated with lower risk and improved prognosis of HCC [88]. Notwithstanding the benefits of surveillance in non-cirrhotic patients with NASH have been questioned by AASLD [8]. Conversely, surveillance is recommended by AASLD in patients with other metabolic diseases like cirrhotic patients with genetic hemochromatosis who have a 20-fold relative risk developing HCC, with an annual incidence of about 3–4 % [89, 90] or patients with stage-4 primary biliary cirrhosis who have about the same incidence of HCC as HCV-cirrhotics [91]. The incidence of HCC in autoimmune hepatitis with cirrhosis is quite low (about 1.1 %/year), not quite making the cut-off of 1.5 % at which HCC surveillance becomes cost-effective [92]. No recommendation was therefore made regarding surveillance in this group and in patients with alpha 1-antitrypsin deficiency, for whom there are insufficient data to accurately assess HCC incidence [93, 94].

22.2.5 Patients on the Liver Transplant Waiting List

Surveillance is endorsed by both AASLD and EASL for Child-Pugh C patients on transplant waiting list with the aim to early detect and manage tumor progression and to help defining priority policies for transplantation.

22.3 Screening Strategy

AASLD, EASL, and APASL share common recommendations for the semiannual surveillance with US of all patients at risk [8, 20, 21]. The choice of APASL of adding AFP as a screening test is not shared by the other associations which consider AFP of inadequate sensitivity and specificity for effective surveillance of HCC and the many small HCCs that do not secrete AFP [95–97]. Indeed, a few early tumors present with abnormal AFP serum levels, including those with the molecular signature of aggressiveness like tumors expressing the epithelial cell adhesion molecule EpCAM [90, 98, 99]. Another important reason for dropping AFP as a surveillance test is the lack of a standardized recall policy for patients without a liver node who have an abnormal AFP test. Finally, cholangiocarcinoma, the second most common primary liver cancer, with a completely different management and prognosis than HCC, may secrete AFP too [91, 92]. However, AFP could maintain a role in the surveillance of selected populations, one above all HBV patients under suppression with nucleotide analogs where confounding due to hepatitis flares is eliminated by effective antiviral therapy (Lampertico et al., unpublished observations).

Alternative serological markers of HCC like descarboxyprothrombin (DCP), glycosylated AFP (L3 fraction to total AFP, alpha fucosidase, glypican 3 (GPC-3), heat-shock protein 70 and DR-70 immunoassay have no added value as screening tests than AFP [100–115]. One possible exception is osteopontin that has been reported to be a more accurate predictor of HCC than AFP; however these observations need to be externally validated [116].

US is the most accurate and widely used test for surveillance. A small HCC on US may take on one of several different appearances, none of which is specific: the smallest lesions may be echogenic, because of the presence of fat in the tumor cells; other may be hypoechoic or show a “target like lesion” appearance. The US sensitivity is between 65 and 80 % with a specificity greater than 90 % when used as a screening test [117]. The widespread popularity of US relies on the absence of risks, non-invasiveness, good acceptance by patients, and relatively moderate cost [115–117]. However, the performance characteristics of US are not ideal in obese individuals with fatty liver disease and cirrhosis. This notwithstanding, US is superior to any serological test and no alternative strategy for surveillance has been adequately tested. Finally, combined use of AFP and US increases detection rate by 6–8 % only, however at the expenses of a substantial increase in costs (80 %) and false-positive rates. Indeed, the false-positive result rates that are 2.9 % for US and 5.0 % for AFP alone, reach 7.5 % for the combination [118].

At variance with AASLD, EASL and APASL, the Japanese Association of the Liver recommends intensified screening

every 3 or 4 months in men with viral cirrhosis or chronic viral hepatitis of increasing age, or with a history of alcohol abuse, since these patients are considered at very high risk of HCC [119]. However, the strategy of intensified screening contrasts with the paradigm that the intervals of screening are not dictated by the level of HCC risk, which may range from 1 to more than 3 % per year, but by the growth rate of the tumor only, which takes 6 months to double its volume, on average [3]. While it is crystal clear that intensified screening aims to identify liver cancer at the smallest size possible in order to optimize treatment, the effectiveness of this policy is largely questioned. In a recent study in France in patients with cirrhosis (mostly alcoholic) who were randomly allocated to standard (6 months) versus intensified (3 months) intervals of screening for HCC [120], during a median period of 47 months the 2 groups of study showed similar rates of cumulative 5-year incidence of HCC nodules (10.0 % vs. 12.3 %), cumulative incidence of HCC $\leq$ 20 mm and 30 mm in diameter, access to curative treatments (62 % vs. 58 %) and liver-related mortality (85 % vs. 86 %). However, the fact that the 5-year cumulative incidence of liver nodules was higher in the 3-month arm (41 % vs. 28 %), clearly heralds a greater economic burden to reach a final diagnosis, which might negatively impact on morbidity and cost utility ratio of intensified screening.

22.4 The Recall Policy

Recall policies consist of a defined algorithm to be activated whenever a surveillance test shows an abnormal result. Any nodule not seen on a prior study should be considered abnormal as an enlarging or changing echo pattern mass, even if previously considered to be benign. The nodular cirrhotic liver poses problems in US interpretation because early HCC can be difficult to distinguish from background nodularity. While a number of cirrhotic nodules can be as large as 2 cm, the majority of nodules smaller than 1 cm are not HCC [121]. Recall is intimately intertwined with the process of making a diagnosis. An accepted rule is to consider any small nodule as an abnormal screening result warranting further investigation [18]. These new nodules should trigger the recall strategy for diagnosis with either non-invasive or invasive (biopsy) criteria. According to both AASLD and EASL guidelines, cirrhotic patients and patients with chronic hepatitis B with a nodule less than 1 cm in diameter detected by US should receive an US examination every 4 months the first year and every 6 months thereafter, until the nodule grows to the point to be diagnosed by either non-invasive criteria or biopsy (Fig. 22.1). CT scan and magnetic resonance imaging (MRI) serve the purpose to demonstrate early arterial enhancement of the nodule and washout of contrast in the portal/venous and delayed phases of the exam [122], which are the radiological hallmarks of HCC. Since US microbubbles are confined to the intravascular space as opposed to iodinated contrast-CT or gadolinium-based MR imaging, where contrast agents are rapidly cleared from the blood pool into the extracellular space, contrast enhancement US (CEUS) may increase the rate of false-positive diagnosis of HCC in patients with an intrahepatic cholangiocarcinoma (ICC), without serving as a staging technique. Thus, CEUS has been dropped from the diagnostic algorithm of HCC endorsed by AASLD and EASL. Along this line, the European Federation of Societies for Ultrasound in Medicine and Biology (EFSUMB) which suggested the typical enhanced pattern for ICC to be a rim-like enhancement (or non-enhancement)

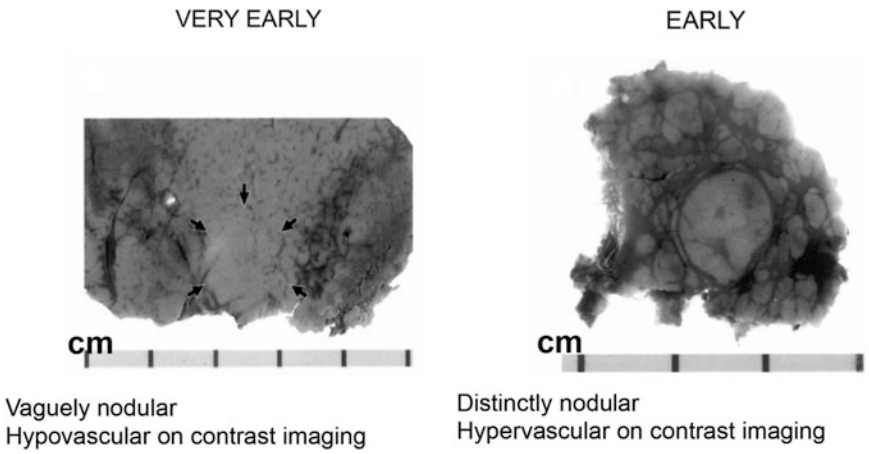

Fig. 22.1 Very early versus early: 5-year survival after resection of 93 % versus 54 %. According to both AASLD and EASL guidelines, cirrhotic patients and patients with chronic hepatitis B with a nodule less than 1 cm in diameter detected by US should receive an US examination every 4 months the first year and every 6 months thereafter, until the nodule grows to the point to be diagnosed by either non-invasive criteria or biopsy. Very early HCC has an indistinct nodular pattern, escapes detection with contrast imaging and has a better prognosis than early HCC. Permission from Elsevier

during the arterial phase followed by hypo/non-enhancement during the portal and delayed phases [123] and APASL endorse dynamic MRI and CEUS for the diagnosis of HCC. Nodular lesions showing an atypical imaging pattern, such as iso- or hypo-vascular in the arterial phase or arterial hypervascularity alone without portal-venous washout, can be better diagnosed by Sonazoid- or Levovist-enhanced US (a second generation contrast enhanced US) and/or SPIO-enhanced MRI to investigate the hepatospecific pattern of the nodules [22].

The AASLD algorithm for investigating nodules between 1 and 2 cm endorses the sequential use of a single imaging technique demonstrating the radiological hallmark of HCC, which has been demonstrated to reduce the need for FNB procedures for the final diagnosis of HCC, without affecting the sensitivity and specificity rates of the recall policy [124–126] (Fig. 22.2). However, the radiological diagnosis of HCC is frequently challenged by false-positive results generated by artero-venous shunts and macroregenerative nodules with dysplastic liver cells. In a retrospective study conducted by Yu et al. [127] in cirrhotic patients with a liver nodule who underwent liver transplant a specificity of 96 and 87 % was found for CT and MRI, respectively, with false-positive imaging results including macroregenerative or dysplastic nodules and non-hepatocellular neoplasms like intrahepatic cholangiocarcinoma (ICC). A lower specificity rate of both imaging techniques was reported in a prospective study of patients under surveillance; because the "typical" vascular pattern was seen in the whole set of high grade dysplastic nodules, whereas a majority of these nodules rapidly progressed toward HCC during the follow-up, outlining the importance of a prompt identification and treatment [128]. Patients with a radiologically undiagnosed liver nodule are indicated to a US guided liver biopsy, which in many instances will disclose the presence of grade-1 HCC endowed with the best prognosis [129]. The strategy of

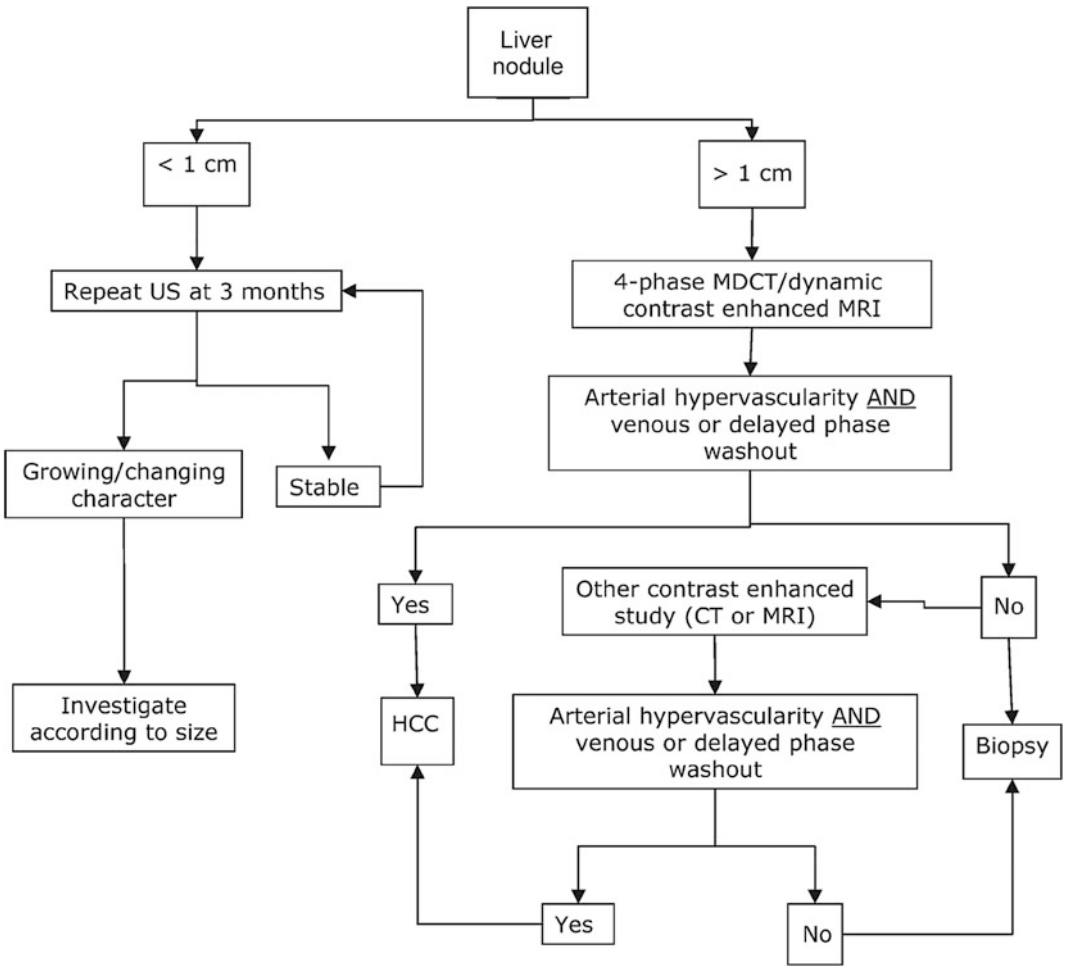

Fig. 22.2 Algorithm for investigation of small nodules found on surveillance in patients at risk for hepatocellular carcinoma [8]. The AASLD algorithm for investigating nodules between 1 and 2 cm endorses the sequential use of a single imaging technique demonstrating the radiological hallmark of HCC, which has been demonstrated to reduce the need for FNB procedures for the final diagnosis of HCC, without affecting the sensitivity and specificity rates of the recall policy. AASLD 2010; Bruix and Sherman. Management of Hepatocellular carcinoma: an update. Hepatology 2011. Permission from Elsevier

Table 22.2 The Importance of Liver Biopsy to Discriminate HGDN from Early HCC

Diagnostic approach		Etiology	HGDN versus HCC	Reference
Histology	Reticulin	HBV/HCV	Stromal invasion (−) versus (+)	Kojiro et al. [132]
Immunostatin	GPC3, HSP70, GS, CHC	Mixed	At least 2: 50 % sens. 100 % spec.	Di Tommaso et al. [133]
PCR	13 genes	Mixed	98 % accuracy	Paradis et al. [134]
	GPC-3 survivin LYVE-1	HCV	94 % accuracy	Llovet et al. [135]
Microarray	120 genes	HBV	100 % accuracy	Nam et al. [136]
	93 genes	HCV	100 % accuracy	Wurmbach et al. [137]

restricting a liver biopsy only to hyper-enhanced nodules or in the presence of synchronous typical HCC to improve the cost utility ratio of screening is questioned by many [130]. Undoubtedly, nodules not diagnosed by radiology require a tight follow-up every 4 months as well as a second biopsy. The risk of seeding should be considered before performing a liver biopsy: in 41 papers specifying the total number of patients biopsied, the median risk of seeding was 2.9 % (range 0–11 %), being lower (0.61–1.4 %) in patients undergoing therapeutic percutaneous procedures [131]. The importance of a liver biopsy rests on its ability to discriminate between HCC and dysplastic macronodules by the exclusion of microscopic stromal invasion [132] (Table 22.2). Immunostaining for GPC-3, and structural and functional analysis of the genetic profile of the nodules may also distinguish between macronodules and HCC but all these approaches likely work better in resected nodules than in tissue cores obtained through a liver biopsy [132]. Immunohistochemistry of more markers may serve the purpose to differentiate HCC from dysplastic nodules, like staining for clathryn heavy chain (CHC) used in addition to HSP70, GPC3 and GS despite the fact that pre-test probability of HCC diagnosis is already high in the set of focal lesions where it was detected [133]. Falsely negative nodules at contrast imaging may account for approximately 20 % of all 1–2 cm in size HCCs [138].

22.5 Efficacy of Surveillance

Surveillance aims to detect small HCCs that are amenable to receive curative treatments, resulting in a significant reduction in liver-specific mortality compared to patients carrying a symptomatic HCC [139–143]. In a meta-analysis of 23 studies in patients with cirrhosis, surveillance for HCC resulted in a 19 % reduction of 3-year mortality [142]. In a retrospective cohort study of 680 patients with a HCC in Taiwan, the receipt of routine or opportunistic (for incidental or non-hepatic purposes) US was associated with a 63 % reduction in mortality compared to the diagnosis of a symptomatic tumor [143]. In the last decade, more than 50 % of all patients in Japan have been diagnosed with a TNM I/II tumor compared to the 1980s, when <10 % of the patients with a HCC was diagnosed at an early stage [144]. In Alaska, a surveillance program of semiannual determinations of serum AFP in HBV carriers led to the identification of curable HCC in 40 % of the affected population, a fact that was perceived as beneficial since prior to AFP screening program the case-fatality rate for HCC in Alaskan natives was 100 %, with an average survival of 3 months only [145]. A randomized controlled study in Shanghai using abdominal US and serum AFP every 6 months to screen individuals with chronic hepatitis and other risks for HCC showed a reduction of the mortality rates in screened versus unscreened population of 83.2 versus 131.5 per 100,000 inhabitants [146]. However, the proportion of patients with cirrhosis was unknown, transplantation was not included among the radical therapies and the compliance of the population to the program was suboptimal (58 %). Notwithstanding all these limitations, the Shanghai study is the only randomized controlled trial to confirm the importance of early diagnosis for improving HCC-related mortality. In Milan, a reanalysis of 112 cirrhotic patients with a HCC detected during a hospital-based surveillance program showed the survival rates to be improved in patients who were treated for a liver cancer detected during the last 5 years of surveillance compared to previous intervals (90 % vs. 55 %, $p = 0.0009$) [147]. Increased survival was attributed to a significant reduction in the mortality rates of treated patients (from 34 to 5 %, $p = 0.003$), due to wider application of curative treatments and improved selection of patients undergoing surgical or ablative treatments. In Taiwan between 1989 and 1998, there was a significant increase in survival among 3345 patients with a HCC during the last 5 years (from 29 to 35 %), that was only in part (34 %) due to advancement in medical care, but mostly (66 %) attributable to early detection [148].

The positive results reported by these observational studies must be interpreted in the context of almost unavoidable potential biases such as lead time bias, i.e., the apparent improved survival that comes from the diagnosis being made earlier in the course of a disease than when the disease is diagnosed because of the development of symptoms or length bias, i.e., the apparent improvement in

survival that occurs because surveillance preferentially detects slow growing and better treatable cancers.

These potential biases notwithstanding, surveillance for HCC is considered a standard of care, not a clinical option. This is clearly perceived by majority of informed patients who believe surveillance to be the only practical approach to improve prognosis of HCC as reported by a survey in cirrhotic patients carried out in three academic centers in Sidney, Australia, who were asked to enter a randomized control trial of surveillance for HCC [149]. Despite appreciating the relevance of a randomized controlled study to determine the applicability, efficacy, and cost-effectiveness of HCC screening, the vast majority of informed responders (98 %) preferred surveillance. One reason for declining randomization is fear of the arbitrary nature of the process and also patients desire to have a more active role in medical decision-making, suggesting that a randomized controlled study of HCC surveillance is nowadays unfeasible in informed patients with a disease like cirrhosis known to predispose to liver cancer. Apparently, cost-effectiveness of screening was less than an issue among patients than it was among physicians, yet most of them (74 %) reported to routinely screen all cirrhotic patients. This contrasts with a population-based study in the USA where 6.6 % of 3903 Medicare patients with HCC were shown to receive regular surveillance prior to diagnosis, only [6], a finding which replicates the low rate of screening uptake (12 %) among hepatitis C infected veterans with cirrhosis [7]. Interestingly, the fact that gastroenterologists, hepatologists, or physicians with an academic affiliation were more likely to perform surveillance than practitioners involved in community-based practices suggests that barriers to screening like limited or outdated knowledge, lack of financial incentives, limited access to appropriate testing and treatment, altogether work against screening effectiveness.

Thus, despite benefits of surveillance for HCC are appreciated by most physicians and patients, surveillance for HCC is not a consolidated practice as it should, even in resource-rich countries. To bridge the chasm of screening for HCC, educational programs advocating screening in risk populations should be implemented targeting both patients and stakeholders in the field, while waiting for a breakthrough in the strategy of screening to occur, which may lead to a switch of screening programs from hospitals to the community, with the aim to improve population's access.

22.6 The Economic Consequences of Surveillance

While the benefits are intuitive, the economic consequences of HCC surveillance strategies are generally poorly appreciated, due to the lack of randomized trials evaluating moderators of treatment outcome like compliance, heterogeneity of liver disease and treatment effectiveness that, in addition to tumor incidence, impact on cost–utility ratio of surveillance. The never-ending argument of cost–utility ratio of surveillance has been analyzed by Markov modeling; moreover in the frame of epidemiological and interventional assumptions which do not necessarily reflect real-life practices. This further underscores the chasm between efficacy and effectiveness of screening for HCC, which may also be inflated by a priori decision to measure cost–utility ratios at less than US$50,000 for quality adjusted life year (QALY) saved. This assumption may conflict with policies of equitability while being influenced by the trends of economy, worldwide [150]. The review and economic analysis published by Coon et al. [151] modeled a population with a diagnosis of compensated cirrhosis who were also eligible to enter a surveillance program. Based on the assumptions used in the model, the most effective surveillance strategy uses a combination of AFP testing and ultrasound at 6-month intervals. Compared with no surveillance, this strategy is estimated to more than triple the number of people with operable HCC tumors at time of diagnosis, and almost half the number who die from HCC. This is a result of the identification of over ten times as many small HCC tumors (less than 2 cm in diameter) and over twice as many medium-sized tumors (between 2 and 5 cm in diameter). Consequently, more tumors are suitable for surgical intervention. Under the conditions of the model, this surveillance strategy would lead to an increase in the percentage of liver transplantations performed for known HCC (as opposed to decompensated cirrhosis) from 8 to 28 %, compared with no surveillance. A cost–utility analysis done in parallel indicates that adding US to 6-month AFP surveillance led to a cost–utility ratio of US$60,000 for QALY gained. Surveillance appeared to be more cost-effective in individuals with hepatitis B-related cirrhosis, potentially due to the younger age at diagnosis of cirrhosis.

22.6.1 How to Optimize Surveillance?

To improve cost-effectiveness of HCC screening, strengthening prediction at individual level through pre-treatment patient stratification by clinical or histological scores has been attempted, yet with uncertain benefits. In a study in Spain, 463 patients were prospectively and randomly included in a program for early diagnosis of HCC [152] based on abdominal US and measurement of AFP levels every 3 or 6 months. In the multivariate analysis, development of HCC was predicted by age 55 years or older, anti-HCV positivity, prothrombin activity 75 % or less, and platelet count less than $75 \times 103/mm^3$. Using these variables to construct a clinical-biological predictive score, two

groups of patients at low (2.3 %) and high risk (30.1 %) of developing HCC in 4 years, were identified.

22.6.1.1 Viremic Patients

Based on a mix of demographic, virological, and clinical features, propensity scores were generated in the NUC era in patients with chronic hepatitis B and therefore they could be used to optimize selection of screenees in HBV hyperendemic areas.

These scores, however, differ from each other in terms of applicability in real life, since REACH-B [153] stands as the only score developed in a community of non-cirrhotic population; conversely, GAG [154] and CU-HCC [155] were obtained in hospital patients, both including the diagnosis of cirrhosis, but only REACH-B and CU-HCC were externally validated.

From a clinical standpoint the three scores shared the merit to accurately identify patients who had remained HCC-free during a surveillance period of 3 years (NPV of 98 %), suggesting their safe use as negative predictors to optimize surveillance programs in an hyperendemic area like China. However, when REACH-B was tested in patients with cirrhosis in the validation study, its prognostic accuracy resulted affected. To overcome the burden of cirrhosis diagnosis, liver stiffness measured by fibroscan was incorporated in CU-HCC, leading to 100 % negative predictive power of the score in a 3-year surveillance period [156]. Unfortunately, all these scores did not optimally perform in non-Chinese populations: when applied to a North American population with HBV, REACH-B was the only model to show a robust negative predictive value for HCC during the first years of surveillance [157].

As expected, risk scores for HCC have been developed in patients with chronic hepatitis C, as well. A score based on age, gender, platelets and AFP was developed more than 10 years ago in Japanese patients with HCV-related cirrhosis and externally validated, providing a frame for stratifying patients into very low, intermediate and high risk groups of developing cancer in a 5 and 10 year period [158]. Unfortunately, the lack of a robust negative predictive power renders this propensity score unfit for optimizing patient selection for screening programs whereas the level of risk does not predict the growth rate of HCC, which in fact is the only parameter to dictate the optimal intervals of screening. More recently, a score has been developed and validated using the REVEAL cohort of asymptomatic anti-HCV subjects in Taiwan, which combines age with laboratory and virology features and diagnosis of cirrhosis [159]. The score succeeded in stratifying subjects in three risk levels independently on viremia, however with an unacceptable 5 % risk of developing HCC in the low risk category. Other scores based on demography, portal hypertension and AFP have been developed in patients with chronic hepatitis C, yet without any external validation, and for this reasons these scores cannot be considered for real-life practice.

22.6.1.2 Non-viremic Patients

Since antiviral therapy does not eliminate the risk of HCC in patients who are chronically infected with HBV while it is an important HCC risk modifier, propensity scores validated in viremic patients need to be separately evaluated in patients with NUC-suppressed viremia to see whether they maintain a robust prediction power, too.

In a comparative study by Wong and associated, all three propensity scores developed in Asia did perform as negative predictors of HCC as they did in viremic patients. In addition, patients with improved GAG and CU-HCC at year two of entecavir therapy had a 50 % reduced risk of developing a HCC during the same time period [160]. This is an important data to refine strategies of surveillance, considering that HCC can only be prevented in two-thirds of patients undergoing 5 years of NUC therapy who were aligned by these scores. In two studies in European patients, the performance of these three Chinese scores was suboptimal, likely consequence of the epidemiological differences existing between Caucasian and Chinese patients with HCC [161, 162]. While the importance of these propensity scores relies on their practicality, we should not forget that in HBV patients undergoing NUC therapy HCC was predicted by patient age, presence of cirrhosis, and diabetes mellitus, suggesting that development of liver cancer in virally infected populations is multifactorial [163]. In the Western world the retrospective analysis of 1666 patients who were long treated with NUCs showed an association between cancer risk and patient age, platelets and liver status. Combining patient age, gender, and platelet count it was possible to elaborate a propensity score named PAGE-B for Caucasian patients under NUC therapy whereby a group of patients with 0 risk of developing liver cancer in a 5-year period of surveillance, could be identified [164].

A propensity score has been developed also to predict HCC in patients with chronic hepatitis C who achieved an SVR to pegIFN based therapy. Using a score based on age, platelet count, AFP, and advanced fibrosis, Chang and co-workers were able to stratify patients into low risk, intermediate risk and high risk of developing liver cancer groups [69]. Unfortunately, the low risk group was burdened by 1.4 % residual risk of developing HCC over a 5-year period of surveillance, a fact that frankly discourages tuning of surveillance strategies by this predictive score system. However, the use of demographic and laboratory criteria makes this propensity score user-friendly and circumvents the need of detecting residual cirrhosis with either non-invasive or invasive procedures.

Currently, none of the propensity scores developed thus far in patients with chronic hepatitis B or C has been

enriched by genetic predictors of tumor susceptibility, possibly because none of studies based on genetic polymorphisms or molecular signatures could identify robust predictors for a molecularly heterogeneous cancer like HCC in at risk populations [165–167].

Propensity scores have been developed to assess HCC risk in both virus etiologies with the aim of optimizing intervals of screening in patients with a robust negative prediction of HCC in a short time period. While prediction is of overwhelming importance to optimize hospital-based surveillance programs with abdominal US, these findings raise the argument whether it can ethically be accepted to deny screening to patient at low risk of cancer therefore jeopardizing patient access to effective radical therapies. Moreover, there is an urgent need to identify HCC predictors in the general population, independently on liver disease etiology that would allow to bring screening for HCC from hospital-based facilities among the community. Such a switch of surveillance strategy might, in fact, improve patient access to screening, thereby resulting in greater survival benefits provided by expanding the number of patients identified with an early HCC.

22.7 Conclusions

A recent study in SEER-13 registries [1] highlighted the emergence of a bounce of epidemiological HCC-related encouraging findings, like the incidence rates of localized-stage HCC increasing faster than rates of regional- and distant-stage HCC combined (8 % vs. 4 % per year). The incidence rates of reported first-course surgery or tumor ablation increased faster than incidence rates of HCC without receiving such treatments (11 % vs. 7 %). Finally between 1975–1977 and 1998–2007, 5-year cause specific HCC survival increased from 3 to 18 %. While this data suggests that HCC survival is improving as a consequence of more patients being diagnosed and treated at early stages, additional progress may be possible through educational programs advocating screening in risk populations while waiting for a breakthrough in the strategy of surveillance to occur which leads to a switch of screening programs from hospitals to the community, with the aim to improve population's access. Finally, although survival benefits of screening are not evidence based, surveillance of patients at risk stands as the only practical approach to reduce HCC-related mortality owing to the remarkable improvement of treatment outcome in patients with early detected tumors compared to those with late discovered, incidental tumors.

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Use of Imaging Techniques to Screen Hepatocellular Carcinoma

23

Satoshi Goshima

Contents

Accurate detection, characterization, and staging of hepatocellular carcinoma (HCC) are the most difficult challenges facing by radiologists and other physicians who caring 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.

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23.1 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.

23.2 Focal Lesions in the Cirrhotic Liver

23.2.1 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. Based on the recent report, confluent hepatic fibrosis is most commonly occurred in the middle hepatic venous drainage area or at the boundary between the medial and anterior segment [3]. On unenhanced CT it is hypodense to liver. In 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. 23.1). MR shows similar morphologic features, including delayed persistent enhancement with IV gadolinium contrast material. More

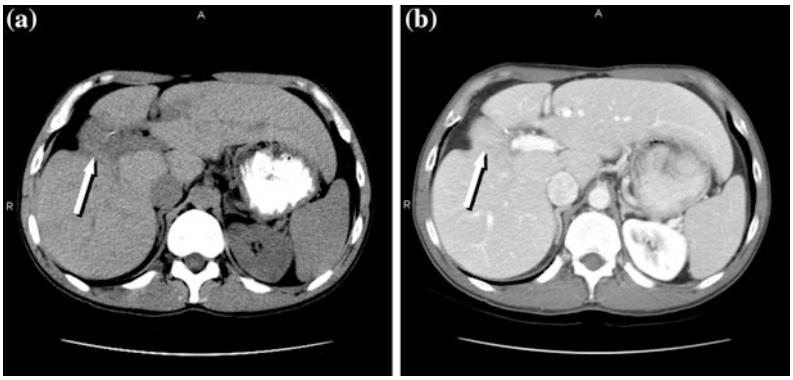

Fig. 23.1 Confluent hepatic fibrosis. **a** Unenhanced CT shows a hypodense lesion (*arrow*) bridging the anterior and medial segments of the liver. **b** Portal venous phase image shows iso-density to the corresponding area (*arrow*). Note the overlying retraction of the hepatic capsule indicating volume loss of this part of the liver. The lesion was isodense to the liver (invisible) on enhanced CT scans

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.

23.2.2 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 (undetectable) on hepatic arterial phase and portal venous phase CT images [4] (Fig. 23.2).

MR detects more regenerating nodules than CT, though it 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. 23.3).

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.

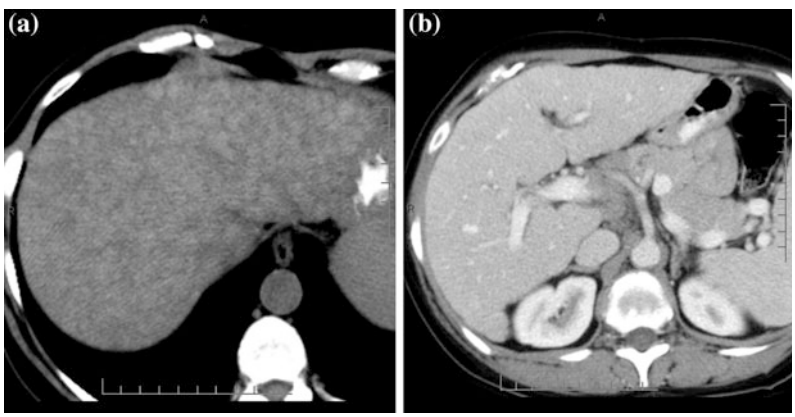

Fig. 23.2 Regenerating nodules. **a** Unenhanced CT demonstrates dozens of hyperdense rounded lesions throughout the liver. Most are about 1 cm in diameter. **b** Enhanced CT (portal venous phase). The nodules become isodense with the liver and can not be detected

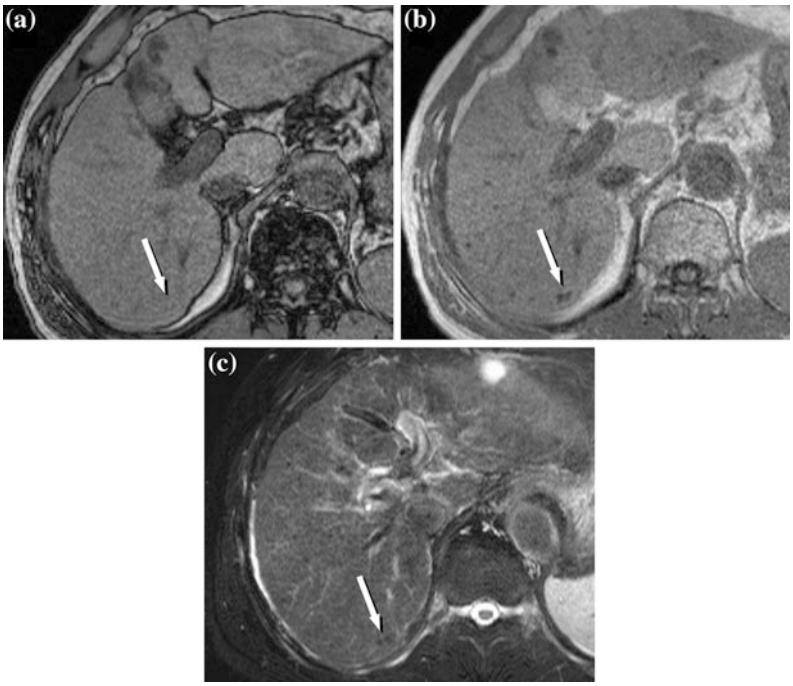

Fig. 23.3 Regenerating nodules. **a** Out-of-phase T1-weighted gradient-echo (TE = 2.2 msec) image shows faint low intensity representing siderotic nodules in segment VI. **b** In-phase T1-weitghted image (4.2 msec) demonstrates darker (hypointense) and blooming subcentimeter lesions to corresponding area. **c** T2-weighted image shows the same lesion is also hypointense to liver

23.2.3 Dysplastic Nodules

Sakamoto et al. and other Japanese investigators have proposed that HCC frequently develops from preexisting 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, low-grade dysplastic nodule (L-DN), high-grade dysplastic nodule (H-DN), and HCC. Analogous to a colonic adenoma evolving into a colonic carcinoma, this theory proposes that some overt HCCs are the end result of a multistep evolution of regenerating nodule to an L-DN than an H-DN and subsequently into HCC. Accordingly, dysplastic nodules are considered premalignant. Dysplastic nodules are found in 11–25 % of explanted livers at transplantation [8–10]. It is reported that cumulative HCC development rates at the first, third, and fifth year were 46.2, 61.5, and 80.8 % for H-DN; 2.6, 30.2, and 36.6 % for L-DN; 3.3, 9.7, and 12.4 % for regenerative nodule, respectively [11].

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 contrast-enhanced ultrasound, dysplastic nodules show arterial hypovascularity in the arterial phase followed by portal perfusion in portal venous phase and isouptake in Kupffer phase [13]. However, we have rarely diagnosed or even correctly suggested the presence of a dysplastic nodule by sonography. Bennett et al. [14] detected only 1.6 % of dysplastic nodules within cirrhotic livers by sonography compared with thin-section explanted liver pathologic results.

Becausee 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, 15]. A diagnosis of dysplastic nodule can be suggested based on a CT finding of a small nodule (≤ 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 [16] (Fig. 23.4). Arterial phase bright enhancement should suggest development of a focus on HCC within a dysplastic nodule, so-called "nodule-in-nodule appearance" (Fig. 23.5). Liver-specific gadolinium contrast agent, including gadobenate dimeglumine and gadoxetic acid disodium as we see later in this chapter, can offer an additional information for the development of HCC within a dysplastic nodule. Dysplastic nodules show iso-intensity on hepatobiliary phase

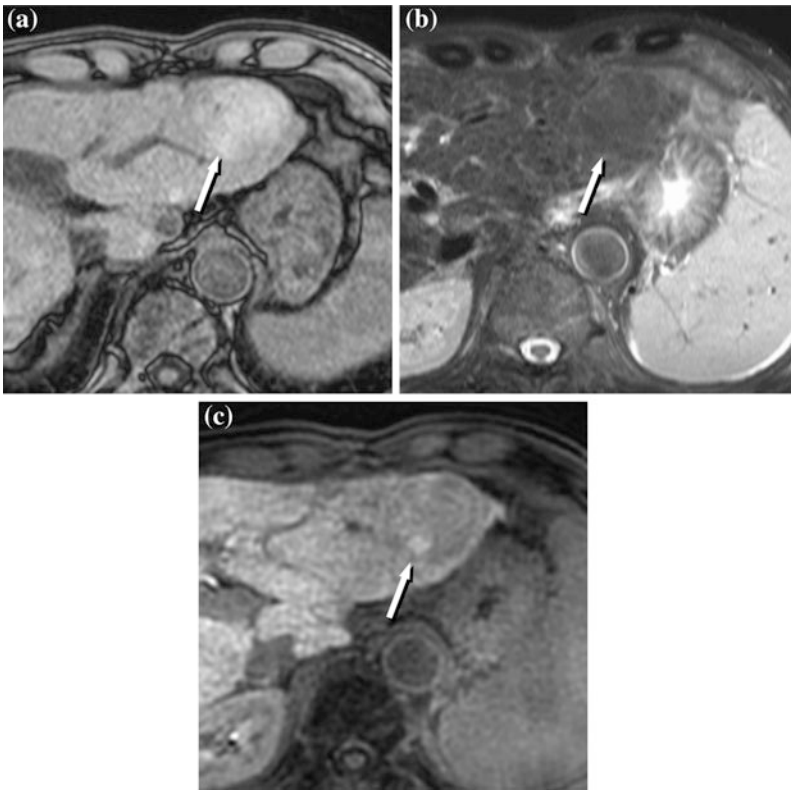

Fig. 23.4 Dysplastic nodules. **a** T1-weighted MR demonstrates 3.0 cm (*arrow*) nodule that is slightly hyperintense to surrounding liver. **b** T2-weighted MR shows the same lesion is slightly hypointense to liver. **c** Gadoxetic acid enhanced hepatobiliary phase image shows the same lesion is isointense to liver

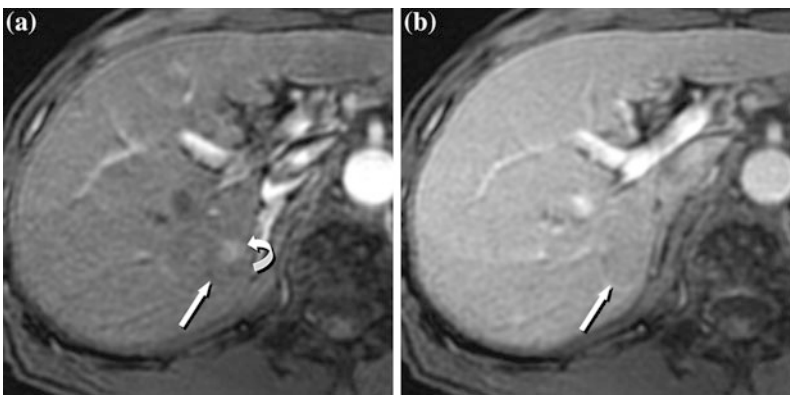

Fig. 23.5 "Nodule-in-nodule appearance" of HCC. **a** Arterial phase MRI shows faint enhancement (*curved arrow*) within the larger hypointense nodule (*arrow*). **b** Portal venous phase MRI shows the entire nodule as iso-intense to liver (*arrow*)

using liver-specific contrast agent, whereas development of HCC typically appears hypointensity [17].

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 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 23.1.

Table 23.1 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

23.3 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 versus 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 is detectable only if set off by a peripheral halo or pseudocapsule [12]. Contrast-enhanced sonography are useful in demonstrating heterogeneous hypervascularity and Kupffer cell function within HCC and may increase the sensitivity and specificity of sonography in diagnosing HCC [18, 19]. HCC is never diagnosed by sonography alone; percutaneous biopsy, usually preceded by CT and MR, 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, multidetector row CT (MDCT) and newer MR pulse sequence including three-dimensional fat suppressed T1-weighted gradient echo have been the mainstay in imaging surveillance of the cirrhotic liver and allow efficient breath-held scanning through the liver prior to contrast administration, as well as during the arterial phase, portal venous phase, and delayed or equilibrium phases of the circulating IV bolus of contrast material [20]. 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. 23.6), and there is ample documentation of the reliability of CT findings in this setting [21, 22].

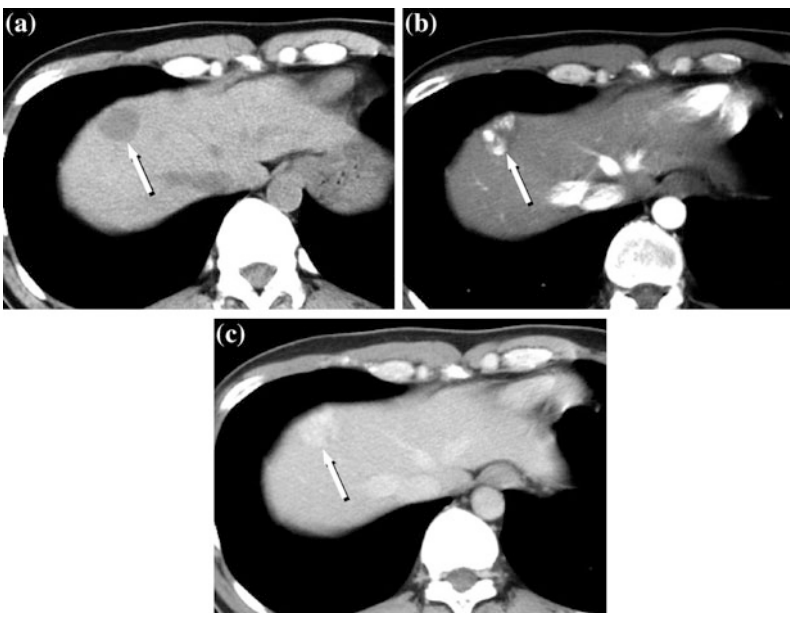

Fig. 23.6 Small cavernous hemangioma. **a** Unenhanced CT. **b** Arterial phase enhanced CT. **c** Delayed phase CT. A 1.5 cm nodule (*arrow*) in the medial segment is isodense with blood vessels on all 3 phases identifying it as an hemangioma rather than HCC

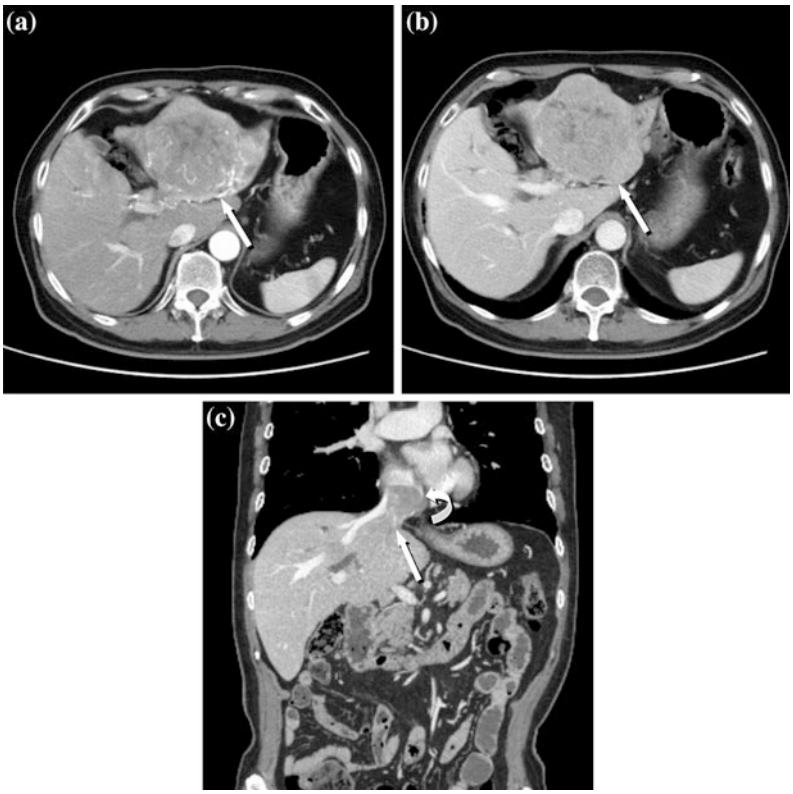

Fig. 23.7 Hepatocellular carcinoma (HCC). **a** Arterial phase CT shows a hypervascular 7 cm tumor (*arrow*). **b** Portal venous phase CT shows the HCC as slightly hypodense to liver (*arrow*). **c** Coronal reformatted portal venous phase CT. The middle hepatic vein are occluded by progressive tumor (*arrow*) continuing to right atrium (*curved arrows*)

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. 23.7). 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.

Aggressive screening should result in the 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, 15, 23] (Fig. 23.8). 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. 23.9). 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 hepatic attenuation difference (THAD) and is usually due to arterioportal shunts or aberrant venous drainage [24, 25]. 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 [26, 27], portal vein obstruction [28], cystic venous drainage [29], or compression effect [30]. Larger segmental or even lobar enhancement differences should prompt close scrutiny for portal venous occlusion or invasion which may result from HCC.

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 hypointense on fat suppressed T1-weighted MR (Fig. 23.8). 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 MRI) 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 [31]. 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, and gadoxetic acid disodium).

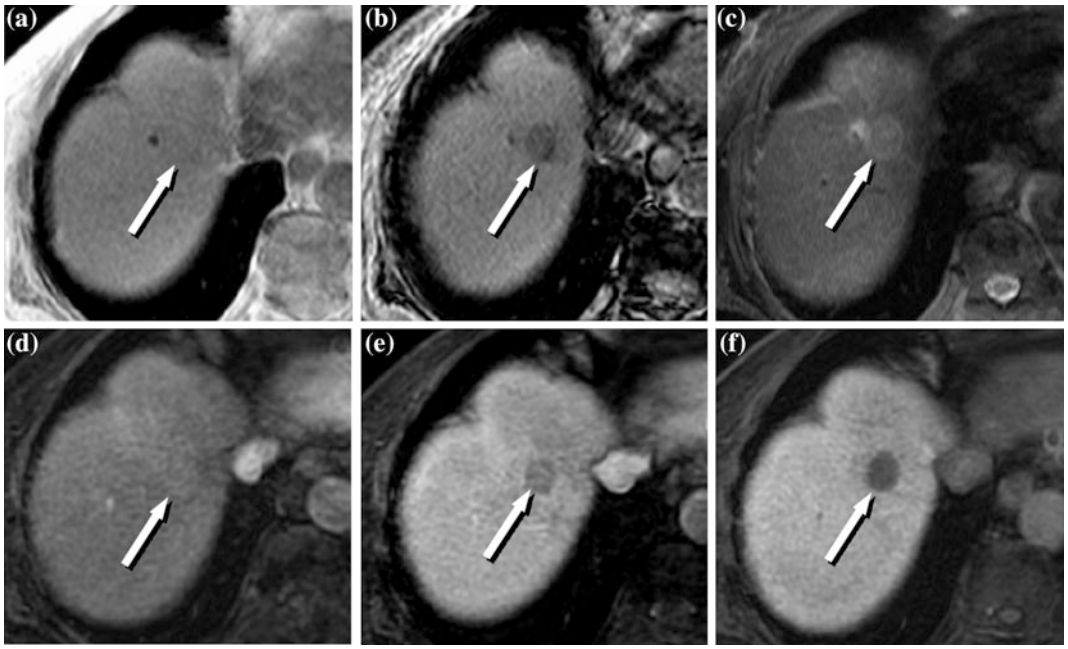

Fig. 23.8 Fat-containing well-differentiated HCC. **a** In-phase T1-weighted image shows little signal difference corresponding to the mass (*arrow*). **b** Out-of-phase T1-weighted image. The mass (*arrow*) shows markedly hypointensity indicating signal suppression due to lipid content of the HCC. **c** Fat suppressed T2-weighted image. The mass shows the same mass is slightly hyperintense to liver. **d** Gadoxetic acid-enhanced hepatic arterial phase image barely detects the mass. **e** Gadoxetic acid-enhanced portal venous phase image shows the same mass is slightly hypointense to liver. **f** Gadoxetic acid-enhanced hepatobiliary phase image shows the same mass is markedly hypointense to liver

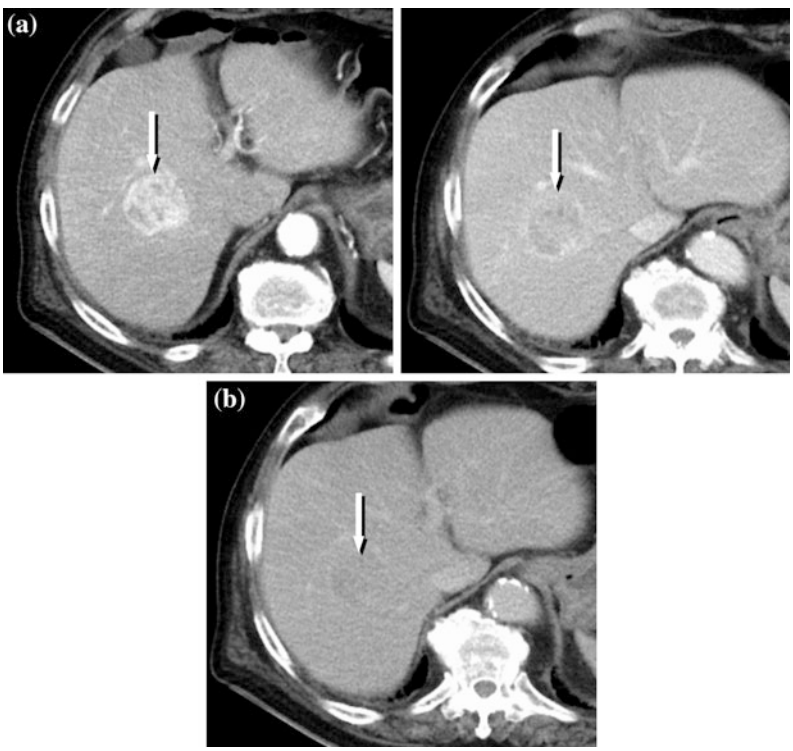

Fig. 23.9 Surveillance for HCC. **a** Arterial phase CT shows slightly heterogeneous hyperdense mass (*arrow*). **b** Portal venous phase CT shows the same tumor (*arrow*) is hypodense to liver. **c** Delayed phase CT. The mass is slightly hypodense to liver

Arterial, portal venous, and delayed phase imaging demonstrate the same hemodynamic tumor characteristics as detailed for CT [31, 32].

Liver-specific MR contrast agent is useful in evaluation of masses within the cirrhotic liver. Gadoxetate disodium (Eovist or Primovist) is a new MRI contrast agent which offers perfusion and hepatoselective properties. It works as an extracellular contrast agent for the first few minutes followed by as a hepatobiliary agent for several minutes [33, 34]. The overt HCC can appear bright enhancement on

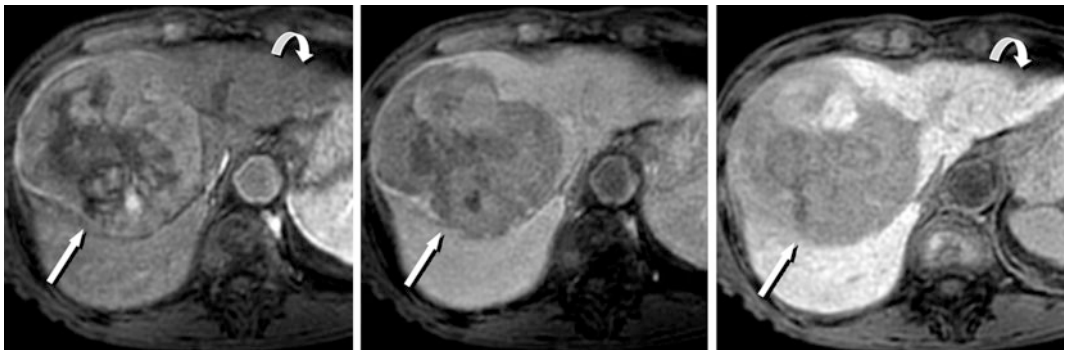

Fig. 23.10 The overt HCC on gadoxetic acid-enhanced MRI. **a** Gadoxetic acid-enhanced arterial phase MR shows a huge hypervascular HCC (*arrow*) and faint tiny enhancement (*curved arrow*). **b** Gadoxetic acid-enhanced portal venous phase MR shows the same huge mass (*arrow*) is hypointense to liver. **c** Gadoxetic acid-enhanced hepatobiliary phase MR shows the same huge mass (*arrow*) is clearly hypointense to liver. Note that tiny hypointense nodule (*curved arrow*) is clearly demonstrated compared to arterial enhancement on A

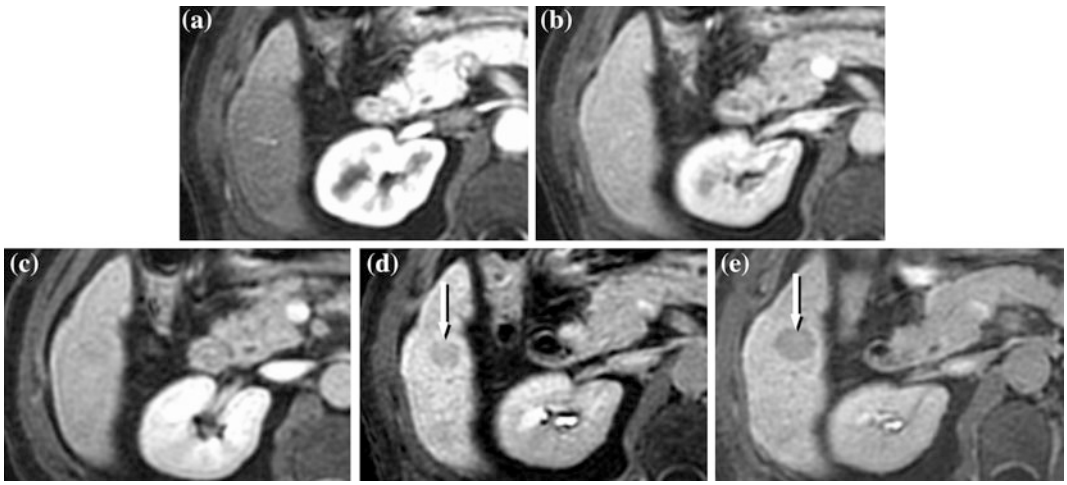

Fig. 23.11 The early HCC on gadoxetic acid-enhanced MRI. **a**–**c** Gadoxetic acid-enhanced arterial (**a**), portal venous (**b**), and delayed (**c**) phase MR barely detect the mass. **d** Gadoxetic acid-enhanced hepatobiliary phase MR clearly detects the hypointense mass (*arrow*). **e** Gadoxetic acid-enhanced hepatobiliary phase MR obtained after 6 months. The mass (*arrow*) has increased in size

arterial phase, washout on portal venous or late-dynamic phase, homogeneous or heterogeneous hypointensity on hepatobiliary phase image (Fig. 23.10). Hepatobiliary phase image offers the best contrast between hypointense HCC and hyperintense surrounding liver tissue. It should be noted that "early HCC" is the current concept introduced by International Consensus Group for Hepatocellular Neoplasms in 2009 [35]. Early HCC is defined as small well-differentiated HCC of vaguely nodular and has a higher 5-year-survival rate compared with progressed HCC. Although most of the early HCCs cannot be detected by CT or MRI using conventional extracellular contrast agent, gadoxetic acid-enhanced MRI is a sole modality to visualize these as hypointense nodules on hepatobiliary phase image without arterial enhancement (Fig. 23.11).

23.4 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. [14] 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 %.

Addley et al. [36] studied 39 patients who had triple-phase MDCT prior to liver transplantation; 29 of these patients had 46 HCC nodules found in the explanted liver. These investigators demonstrated 65–75 % sensitivity for detection of overall HCCs but the sensitivity decreased to 48–57 % for the lesions of size $\leq$20 mm.

Kakihara et al. [37] performed gadoxetic acid-enhanced MRI in 15 patients, including 36 HCC nodules, who had living related-liver transplantation and pathological correlation of the explanted liver with the MR interpretation. Although these investigators included relatively small HCCs in their study (size range, 0.5–6.3 cm; median size, 1.3 cm), sensitivity, specificity, and accuracy for the detection of HCC are 47–61, 98–99, and 83–86 %, respectively.

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.

23.5 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 [38] (Fig. 23.12). 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) [39, 40].

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 [41]. 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 % [42, 43].

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 4-phase dynamic CT or MRI 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 [44, 45]. The Barcelona panel has set a goal of detecting tumors below 3 cm in diameter and recommends surveillance at 6-month intervals, while Japanese consensus guideline are much more aggressively recommending sonography and tumor marker measurements every 3–4 months, dynamic CT or MRI 6–12 months, and gadoxetic acid-enhanced MRI if the abnormality was detected by prior examination [46]. This surveillance

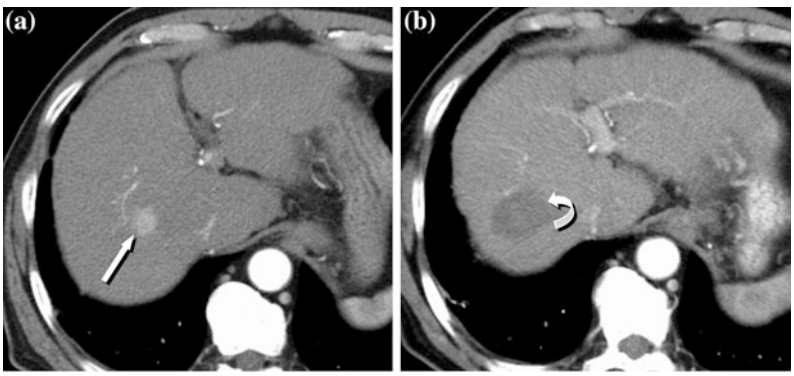

Fig. 23.12 Small HCC treated with radio frequency ablation. **a** Arterial phase CT shows a 1 cm hypervascular nodule (*arrow*). **b** Following percutaneous RF ablation under ultrasound guidance the ablation defect is shown (*curved arrow*), with no viable tumor on enhanced CT

protocol is applied to patients with established cirrhosis; for patients with chronic hepatitis without established cirrhosis, sonography and tumor marker measurement are recommended every 6 months in this guideline.

It is clear 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 the American Association for the Study of Liver Disease (AASLD) also recommends that sonography will be a cost-effective screening tool. Since American cirrhotic patients are also more likely to be larger and to have hepatic steatosis, factors which further limit the accuracy of sonography, 4-phase dynamic CT or contrast-enhanced MRI are preferable for further examination.

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, with extracellular or liver-specific contrast agent, may be the single most accurate imaging test assuming optimized technique and expert interpretation.

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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Ultrasound of Hepatocellular Carcinoma: The Important Contribution of Contrast Enhancement

24

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Contents

24.1 Introduction

The noninvasive diagnosis of hepatocellular carcinoma (HCC) relies heavily on imaging-based primarily on sequential changes in the intranodular blood supply during the process of hepatocarcinogenesis [1]; regenerative nodules (RN) show similar blood supply to normal liver, borderline lesions show wide variations of blood supply [2] and typical HCC are supplied by abnormal neoplastic arteries alone. Once a focal hepatic nodule is detected during HCC surveillance typically with ultrasound (US), a diagnostic imaging test is performed. While contrast-enhanced CT or MRI is most commonly selected as the diagnostic test, contrast-enhanced ultrasound (CEUS) using a microbubble contrast is an excellent choice that has several advantages over CT or MRI including a real-time demonstration of continuous hemodynamic changes of liver tumors, a purely intravascular contrast material, availability in patients with renal failure, excellent patient compliance, and repeatability in short intervals.

Management strategy for HCC is often decided in multidisciplinary consensus meetings including physicians from several different specialties. The role of imaging in the diagnosis and staging for HCC is crucial to determine the management plan. Recent practice guidelines for HCC provide recommendations for the diagnostic algorithm for newly detected nodules at HCC surveillance [3–5]. The application of the imaging test varies depending on the size of the nodules. For very small lesions (<1 cm in size), follow-up with US scan is usually recommended in 3 months as further imaging tests may not be reliable for the diagnosis. For lesions of 1 cm or larger, multiphasic contrast-enhanced CT, MRI or CEUS is usually performed as a diagnostic test. As the imaging diagnosis of small nodules of 1–2 cm in size can be particularly challenging, a multimodality approach is often needed [6]. Borderline lesions, i.e., high-grade dysplastic nodule (DN) and well-differentiated HCC, often show indeterminate imaging findings and imaging may not be reliable to differentiate

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between the two [2]. Biopsy is performed only when imaging findings are indeterminate.

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 [7]. CEUS is particularly useful for detecting arterial-phase hypervascularity of HCC utilizing the real-time evaluation of the lesion perfusion. CEUS is an excellent modality to assess post-ablative therapy for HCC. CEUS is also useful to differentiate between malignant and benign venous thrombosis in patients with HCC [8], which is often critical to determine the management plan.

In this chapter, we review the CEUS techniques and typical CEUS imaging features of HCC and other cirrhosis-related nodules. We also discuss the role of CEUS in the algorithms for the diagnosis and staging of HCC and in monitoring therapeutic responses to local ablation therapy.

24.2 Contrast-Enhanced Ultrasound Techniques

US contrast agents consist of microbubbles of perfluorocarbon gas stabilized by a protein, lipid, or polymer shell. The microbubbles are sufficiently small and stable to traverse the pulmonary and cardiac circulations following peripheral venous injection. The microbubbles disappear as the gas diffuses through the thin shell, with a typical half-life of a few minutes in blood and there is no renal excretion. There are a few different types of microbubble contrast agents that are commercially available. Presently, Definity (Lantheus Medical Imaging, Billerica MA) and SonuVue (Bracco, Milan, Italy) are most widely used. Microbubbles are approximately the same size as red blood cells and cannot move through the vascular endothelium into the interstitium; therefore, they are true blood pool agents [9]. Sonazoid (Daiichi), which is most actively used in Japan, shows similar vascular enhancement but is taken up by Kupffer cells in the late phase [10]. In our experience of using Definity for over 12 years, patient acceptance has been very high and there have been no serious adverse events. A large retrospective study from Europe using SonoVue reported 0.0086 % incidence of serious adverse events without any fatality among 23,188 examinations [11]. Microbubble contrast agents are approved for radiologic use in more than 50 countries, including the European Union, Canada, and many Asian countries.

Definity and Sonovue are both approved for cardiac use in the United States, and on April 2016, the FDA approved Sonovue for liver mass characterization for adults and children. Sonovue is marketed as Lumason in the USA. CEUS requires a contrast imaging mode that is available on most high-end commercially available ultrasound systems. Low mechanical index (MI) contrast-specific mode is used to visualize the microbubbles continuously while suppressing signals from tissue. A dual-imaging mode (Fig. 24.1), which enables simultaneous real-time display of contrast-specific mode and the gray-scale mode, is essential for scanning small liver lesions. Typically, the contrast agent is injected manually through a three-way stopcock, followed by a 5-mL saline solution flush. Continuous scan with video acquisition is performed in the arterial phase (usually <30 s after saline flush) to evaluate the real-time enhancement pattern of the liver lesion. Then the liver lesion is intermittently scanned typically every 30 s for 4–5 min to minimize inadvertent microbubble destruction. Sweeping of the entire liver can be performed in the late phase to detect any additional washout lesions. Slightly higher MI along with a larger amount of microbubbles can be used for deep seated lesions or lesions within an attenuating fatty liver.

The first injection usually includes a stationary field of view to include the lesion of interest and the adjacent liver, both observed 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 phase to look for any further abnormalities. Injections are typically repeated 2–3 times to obtain images of the same lesion or to evaluate a different lesion. Each injection is separated by 3–5 min. High MI frames can be used to disrupt microbubbles and evaluate the pattern of refilling of the microbubbles in the scanning plane. This may be optimized by using bubble tracking technology which is called maximum-intensity projection, most optimally used to show the filling pattern and vascular morphology of hypervascular liver tumors (Fig. 24.2) [12].

24.3 Differential Diagnosis of Nodules in Liver Cirrhosis

Typical HCC are supplied by abnormal neoplastic arteries alone and show hyperenhancement relative to the liver in the hepatic arterial phase (hypervascularity) and hypoenhancement in the late phase (washout) (Fig. 24.3) [13–15]. There are irregular dysmorphic arteries within the tumor often visualized in large HCC at the early arterial filling phase (Fig. 24.2). Arterial-phase enhancement pattern of HCC is usually homogeneous in small lesions (Fig. 24.3) and tends to be heterogeneous in large lesions with or without non-enhancing areas representing necrosis (Fig. 24.2). Peripheral rim-like enhancement is uncommon in HCC. A nodule-in-nodule pattern is occasionally seen when there is a hypervascular HCC focus developing within an underlying DN or well-differentiated HCC (Fig. 24.4) [2, 16]. The hypervascular focus in HCC usually shows washout and

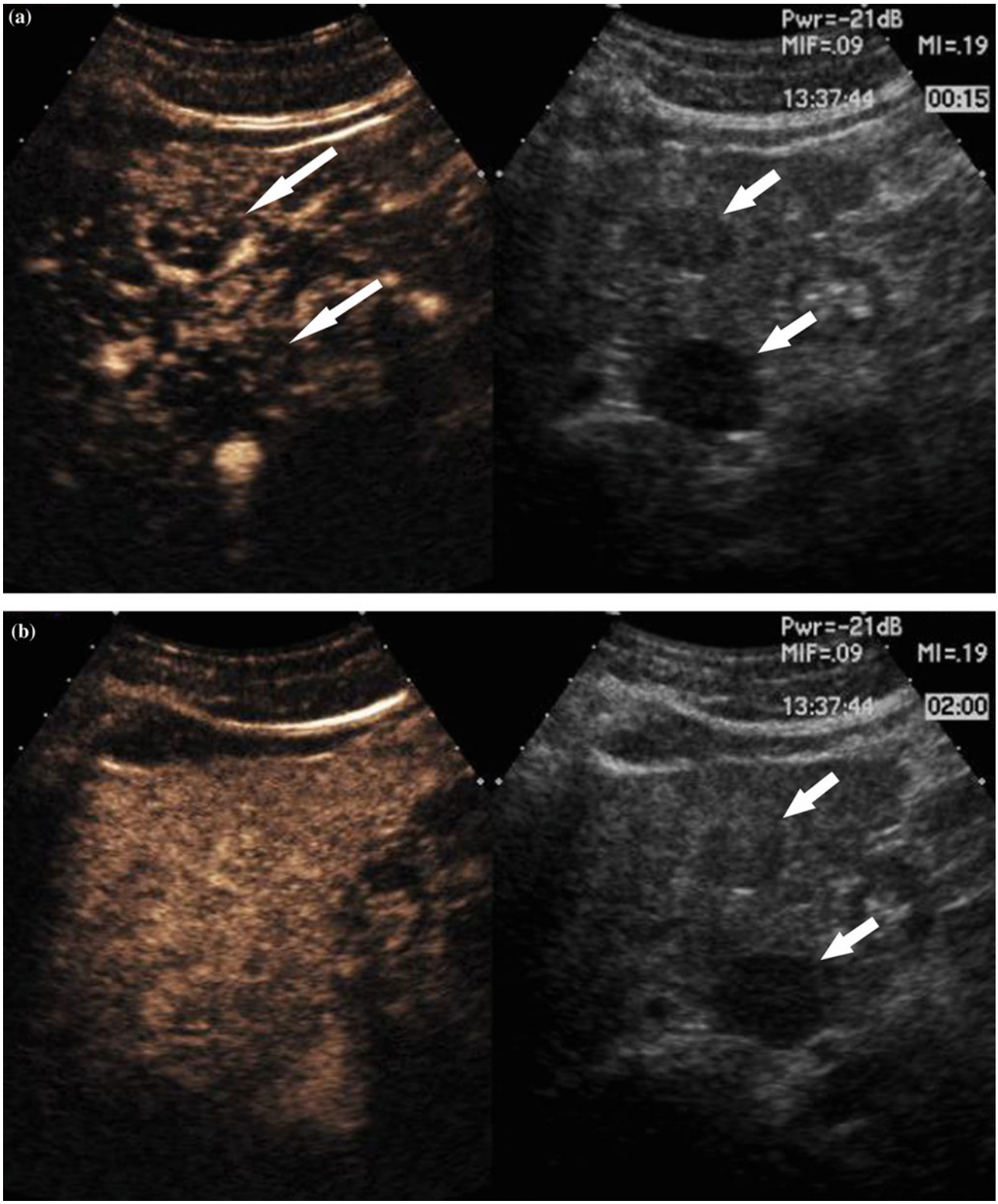

Fig. 24.1 Well-differentiated HCC in a 73-year-old man with hepatitis C. **a** A dual-imaging mode CEUS displays contrast-specific mode on the left and gray-scale mode on the right simultaneously. There are two hypoechoic masses (*short arrows*) in the liver on gray-scale mode that are slightly hypoechoic (hypovascular) relative to the liver (*long arrows*) on contrast-specific mode in the arterial phase. **b** In the portal venous phase, the hypoechoic masses (*short arrows*) on gray-scale mode are not seen on contrast-specific mode as they are isoechoic to the liver at 2 min

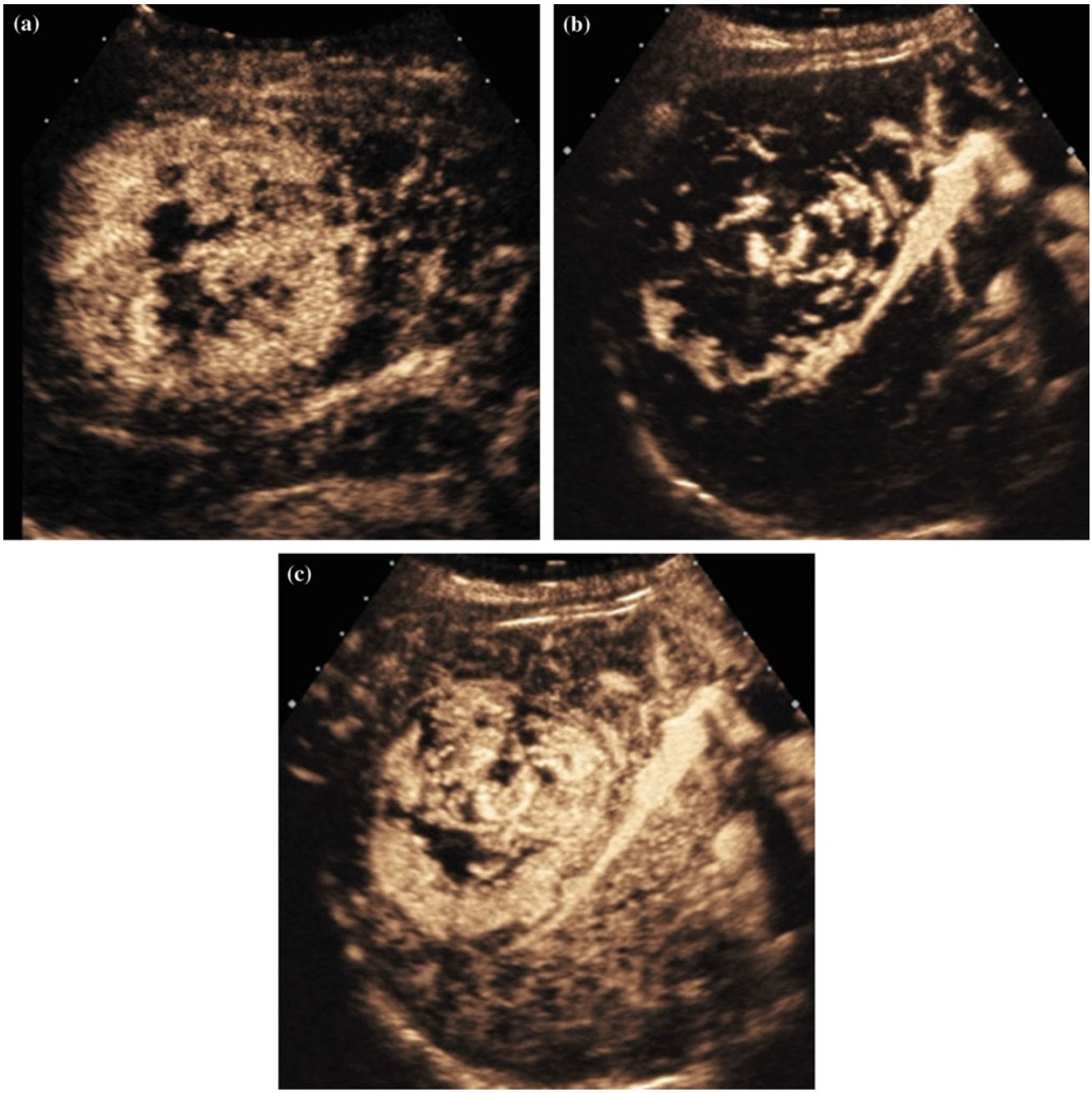

Fig. 24.2 HCC in a 83-year-old woman with hepatitis B. **a** CEUS scan in the arterial phase shows a large hypervascular mass in the liver with heterogeneous enhancement and non-enhancing areas representing necrosis. **b**, **c** Two maximum-intensity projection CEUS images after microbubble disruption by using high MI frames demonstrate irregular, dysmorphic, neoplastic arteries within the mass that are not seen on regular CEUS image (**a**)

should not be confused with a nodular enhancement in hemangioma, which progresses centrally over time and shows sustained enhancement without washout.

Detection of arterial-phase hypervascularity is crucial to make a noninvasive diagnosis of HCC. CEUS allows a real-time assessment of arterial-phase enhancement, eliminating the issue of inappropriate arterial-phase timing. CEUS often detects arterial-phase hypervascularity when CT or MRI fails to show this because of incorrect arterial-phase timing [17]. One of the most common indications of CEUS is to evaluate small, indeterminate, non-hypervascular nodules seen on CT or MRI. CEUS is often able to diagnose HCC by detecting hypervascularity in some of these lesions (Fig. 24.5), preventing an invasive biopsy [17–19]. It is often difficult to assess arterial-phase hypervascularity in markedly hyperintense nodules on unenhanced T1-weighted

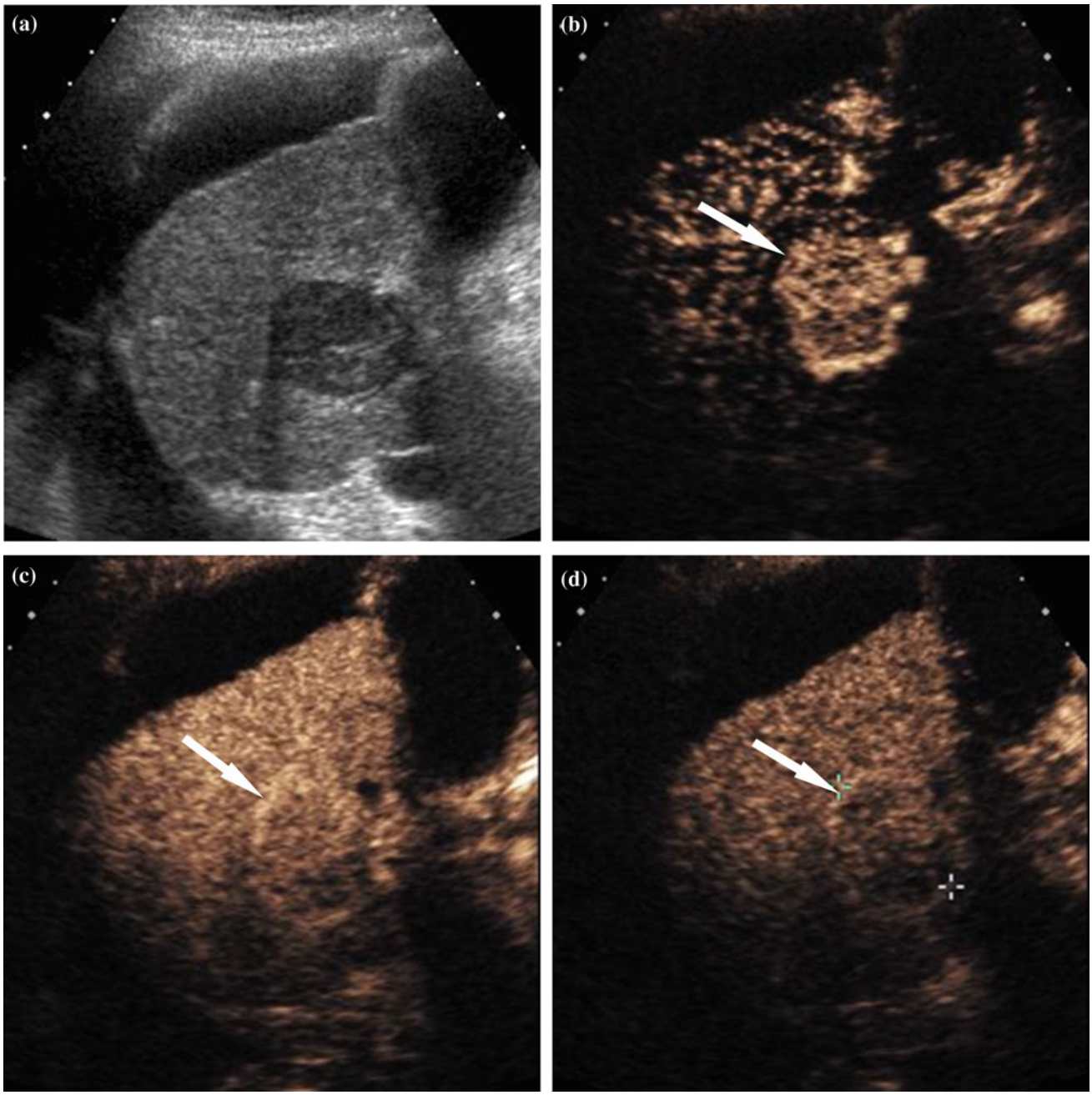

Fig. 24.3 Typical hypervascular HCC with late, mild washout in a 70-year-old man with hepatitis C. **a** US scan shows a hypoechoic, solid mass in the liver. The liver is cirrhotic with a nodular surface and there is a large amount of ascites. **b** CEUS scan in the arterial phase at 20 s shows homogeneous hypervascularity of the mass (*arrow*). **c** The mass (*arrow*) is isoechoic to the liver CEUS scan at 3 min. **d** The mass (*arrow*) shows mild washout at 5 min

MR images, especially when there is an iron overload in the underlying liver with marked hypointensity (Fig. 24.6). CEUS can be used as a problem-solving method as the nodules are completely anechoic on contrast-specific mode before microbubble injection.

Hypoenhancement or "washout" in the late phase is also an essential imaging feature for diagnosing HCC as typical HCC lack portal venous supply. Washout is more consistently seen on CEUS than CT or MRI due to the differing characteristics of the contrast material. CT or MRI may not show washout in malignant tumors with large extracellular space and high vascular permeability as the contrast material leaks and accumulates into the tumor interstitium, whereas microbubbles in CEUS are purely intravascular and show washout (Fig. 24.7) [17]. The intensity of enhancement of HCC in the late phase, however, generally decreases more

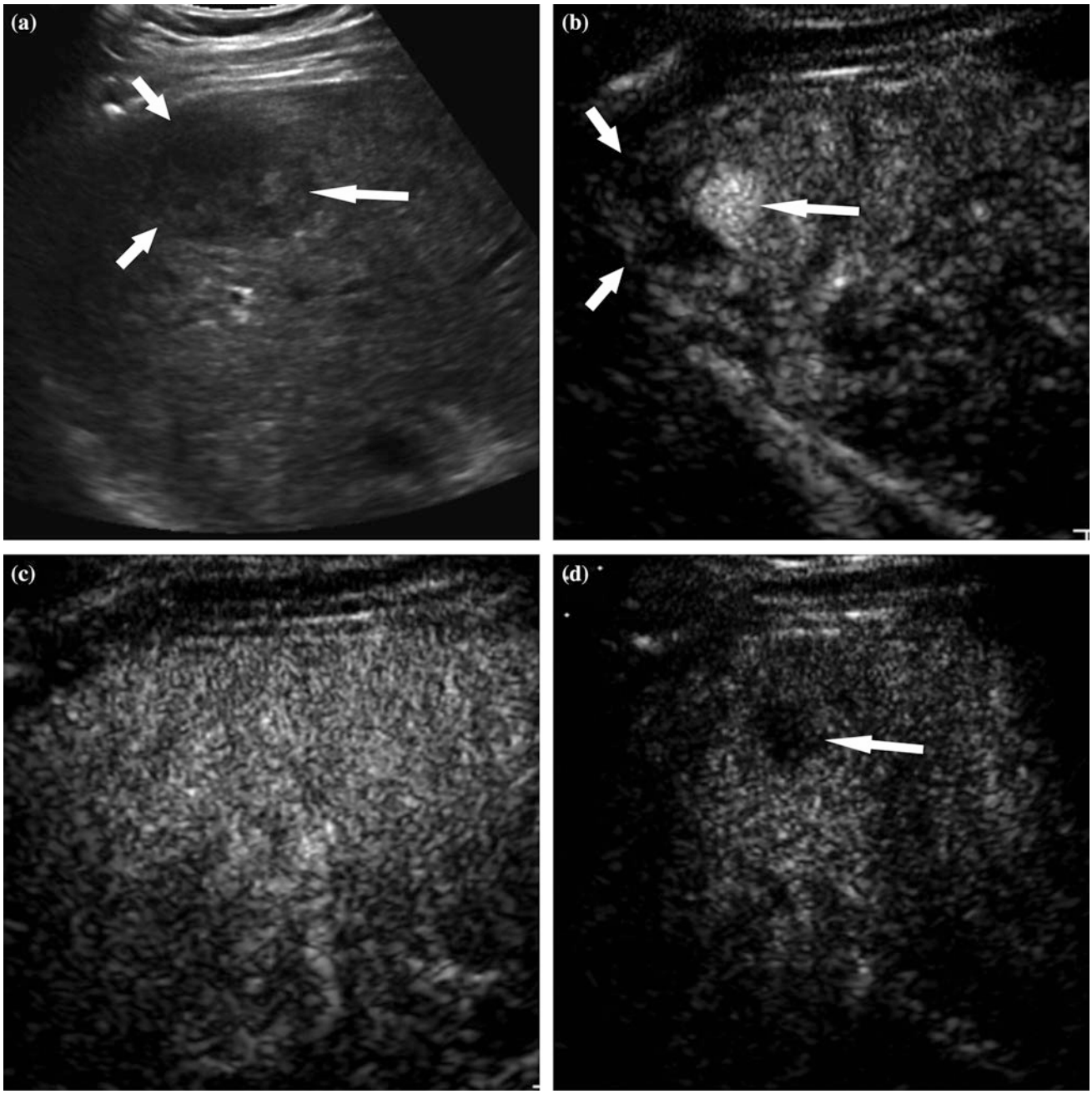

Fig. 24.4 HCC with nodule-in-nodule pattern in a 60-year-old man with hepatitis B. **a** US scan shows a hypoechoic mass (*short arrows*) with a slightly hyperechoic focus (*long arrow*) in the liver. **b** CEUS scan in the arterial phase at 8 s shows hypovascularity of the mass (*short arrows*) with a hypervascular focus (*long arrow*), a nodule-in-nodule pattern. **c** The mass is not seen because of isoechogenicity at 90 s. **d** Focal washout (*arrow*) is only seen at 3 min where a hypervascular focus was seen in the arterial phase (**a**)

slowly than cholangiocarcinoma or metastasis. Washout in HCC often begins later than 90 s after injection (Figs. 24.3 and 24.5) whereas metastases or intrahepatic cholangiocarcinomas consistently show rapid washout beginning before s 60 s (Fig. 24.8) [20–22]. In our study of 115 hypervascular HCC [23], only 50 % showed washout by 90 s. Extended evaluation over 4–5 min is important to characterize HCC by demonstrating "eventual" washout (Figs. 24.3 and 24.5). Washout timing is related to the pathologic differentiation of HCC: well-differentiated HCC tends to show later washout or no washout, whereas poorly differentiated HCC tends to show more rapid washout [23]. Therefore, no washout for 4–5 min should not be considered for a diagnostic finding of a benign lesion (Fig. 24.9). In fact, most new hypervascular nodules on CEUS detected during HCC surveillance are HCC regardless of washout if the nodules do not show the

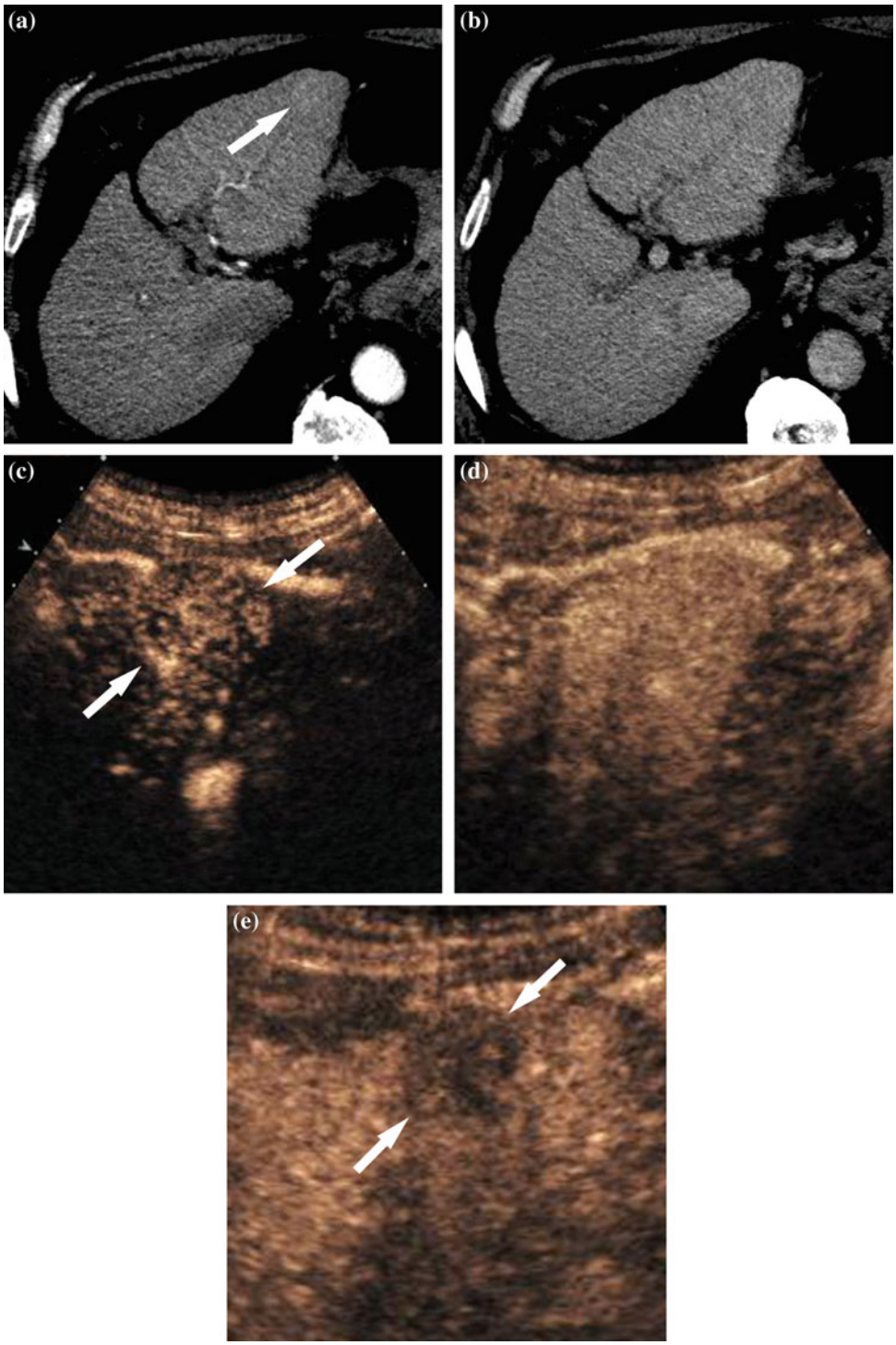

Fig. 24.5 HCC in a 65-year-old woman with hepatitis C. **a** CT scan in the arterial phase shows a subtle hyperattenuating lesion (*arrow*) in the left lobe of the liver. **b** The lesion is not seen in the delayed phase. CT findings of the liver lesion are indeterminate. **c** CEUS scan in the arterial phase at 15 s shows a hypervascular mass (*arrows*) in the liver. **d** The mass is not seen due to isoechogenicity to the liver at 150 s. **e** The mass (*arrows*) shows clear washout at 270 s. CEUS findings are diagnostic of HCC

appearance of hemangioma [24]. However, a biopsy is needed to confirm HCC for hypervascular nodules without washout.

There is a small subset of HCC with no arterial-phase hypervascularity, including particularly those that are well differentiated. In our study of 112, HCC that were evaluated with CEUS, 23/112 (21 %) were well-differentiated and 9/23 (39 %) were not hypervascular [23]. These lesions occasionally show a transient hypoenhancement in the arterial phase followed by gradual enhancement and the lesions become isoechoic relative to the normal liver in the late phase (Fig. 24.1). These hypovascular HCC cannot be reliably differentiated from DN by imaging findings alone, requiring biopsy for confirmation.

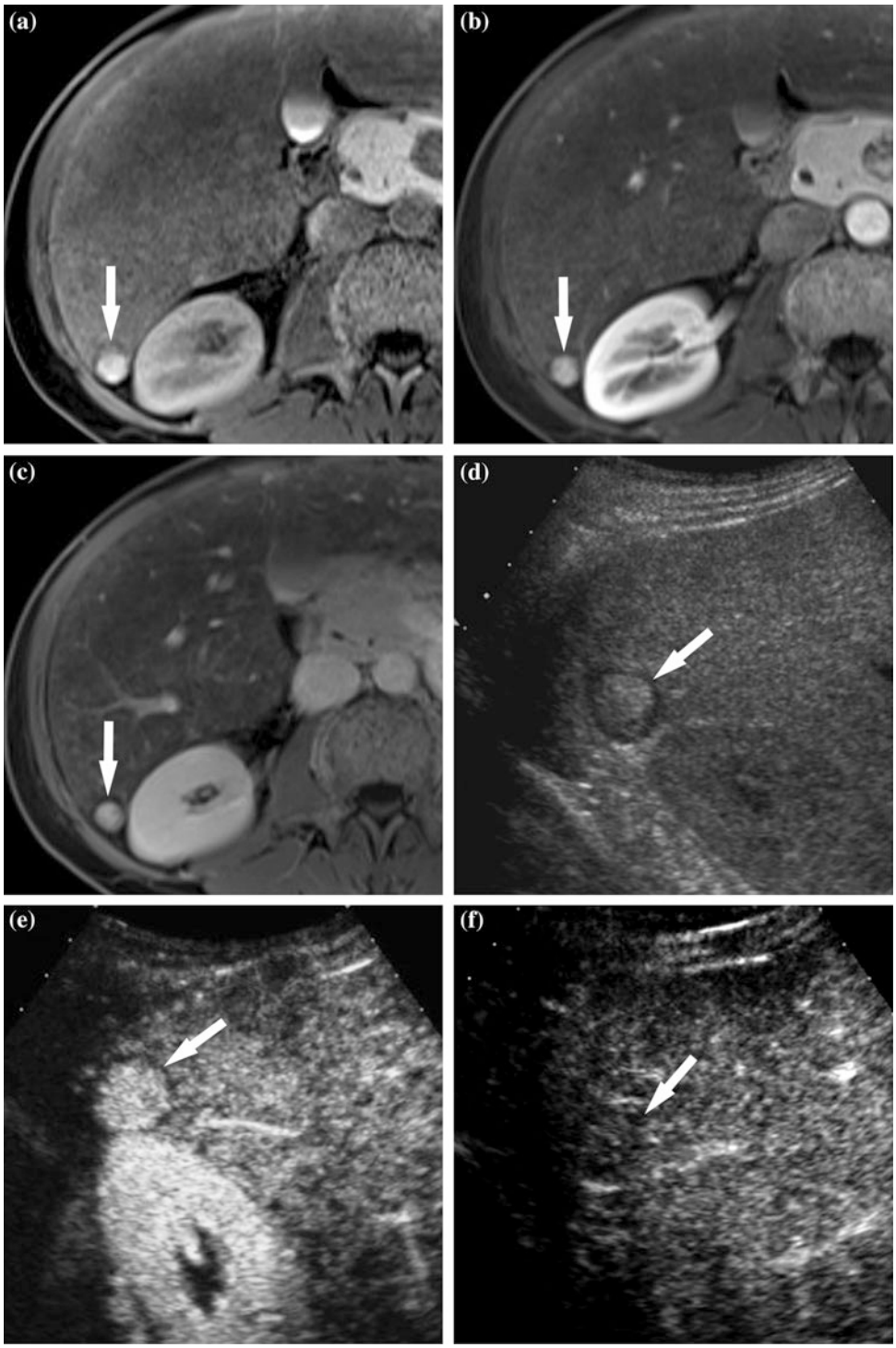

Fig. 24.6 HCC in a 43-year-old man with thalassemia and secondary hemochromatosis. **a** Unenhanced T1-weighted MR scan shows a brightly hyperintense nodule (*arrow*) in the liver. Underlying liver is diffusely hypointense due to hemochromatosis. **b**, **c** The nodule (*arrow*) is hyperintense in the arterial-phase (**b**) and delayed phase (**c**) MR images. The findings are indeterminate as the evaluation of arterial-phase hypervascularity and washout is challenging. **d** US scan shows a slightly hyperechoic nodule with thin hypoechoic halo (*arrow*). **e** The nodule (*arrow*) is hypervascular in the arterial phase at 7 s on CEUS. **f** The nodule (*arrow*) shows slight washout at 135 s

Liver cirrhosis related to viral hepatitis is also identified as a risk factor for development of intrahepatic cholangiocarcinoma (CC) although the incidence of CC is much lower than that of HCC. Therefore, small CC is infrequently detected during HCC surveillance. Accurate imaging differentiation between CC and HCC, however, is important because the treatments for the two conditions are different. On CEUS, small CC usually shows arterial-phase hypervascularity and washout similar to HCC [25]. However, the diagnosis of intrahepatic CC can be suggested by CEUS in most cases, by demonstrating rim-like arterial-phase enhancement (Fig. 24.10) and/or rapid washout (<60 s) and/or a punched-out appearance of the washout at its first observation (Fig. 24.8) [20–22, 26]. Punched-out washout is

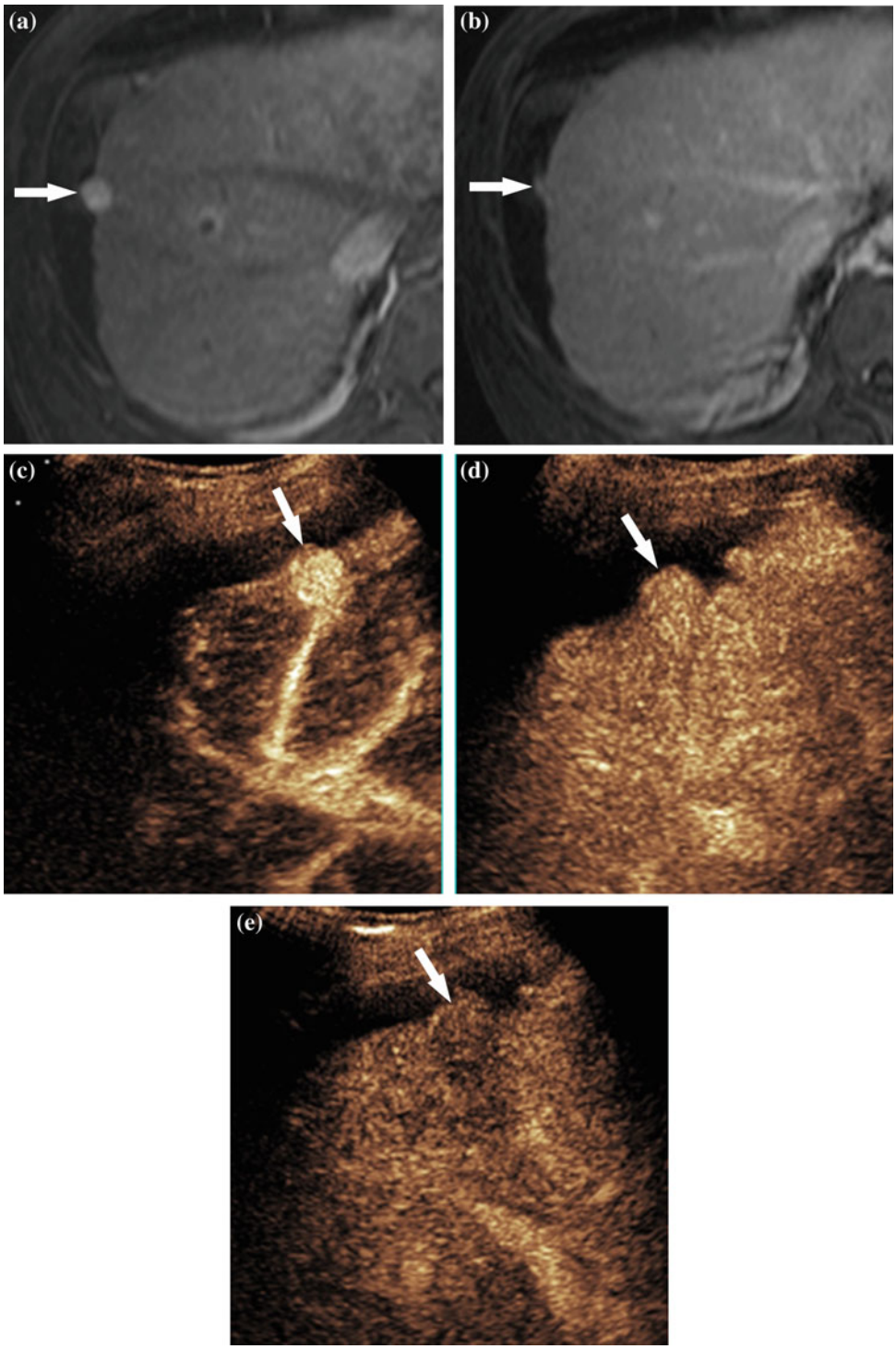

Fig. 24.7 HCC in 56-year-old man with hepatitis B. **a** MR scan in the arterial phase shows an exophytic hypervascular nodule (*arrow*) in the liver. **b** The nodule (*arrow*) is isointense to the liver in the delayed phase without washout. **c** The nodule (*arrow*) is hypervascular in the arterial phase of CEUS. **d** The nodule (*arrow*) is isoechoic at 3 min. **e** The nodule (*arrow*) shows washout at 5 min, confirming the diagnosis of HCC

not commonly seen in HCC and, if observed, follows an initial observation of weak washout. Combined CC and HCC in a single liver mass is rare and the clinical and imaging findings are determined by the dominant proportion of the histological component [27]. CEUS findings can be similar to CC when the CC component is dominant. Hepatic capsular retraction near the liver mass (Fig. 24.11) is a suggestive finding of CC [28] as it is rarely seen in HCC. Biopsy should be performed when these unusual enhancement patterns for HCC are observed on CEUS.

RN form the essential component of a cirrhotic liver and are small and usually do not stand out on imaging. On grayscale US, numerous RN in cirrhotic livers are typically seen as coarse and heterogeneous liver with a nodular

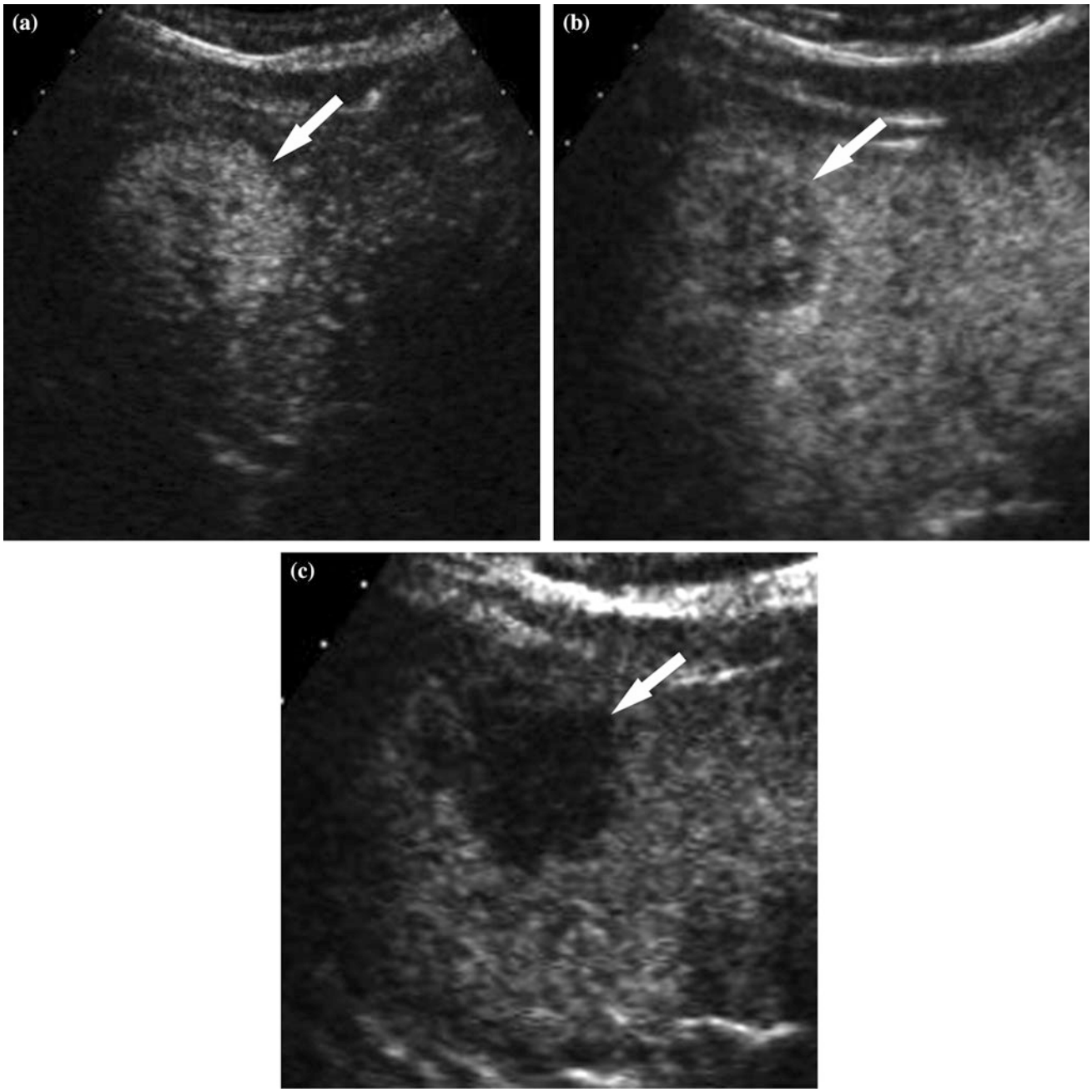

Fig. 24.8 Intrahepatic CC in a 58-year-old man with hepatitis B. **a** CEUS scan in the arterial phase at 17 s shows a mass (*arrow*) with diffuse arterial-phase hypervascularity. **b** CEUS scan at 28 s still in the arterial-phase time frame shows washout (*arrow*). **c** CEUS scan at 280 s shows marked washout with a punched-out appearance (*arrow*)

surface. Most RN are isoechoic to the parenchyma during all phases on CEUS. As DN have more histological atypia, abnormal arteries increase while normal arterial and portal supply decrease. The arterial and portal supplies to DN, therefore, are variable and inconsistent [29] (Fig. 24.12). As there is significant overlap of vascular supply between high-grade DN and well-differentiated HCC, imaging differentiation between the two is challenging and often unreliable [2]. Biopsy is often performed for the differentiation; however, the differential diagnosis in small needle biopsy specimens can be also challenging due to histological heterogeneity within the nodules. In the setting of a competing potentially fatal disease (i.e., cirrhosis), imaging follow-up instead of invasive biopsy is often applied for evaluating small borderline liver lesions [30].

Hemangiomas are frequently detected during HCC surveillance. In our study [31], 43/184 (23 %) of newly detected nodules at HCC surveillance were hemangiomas.

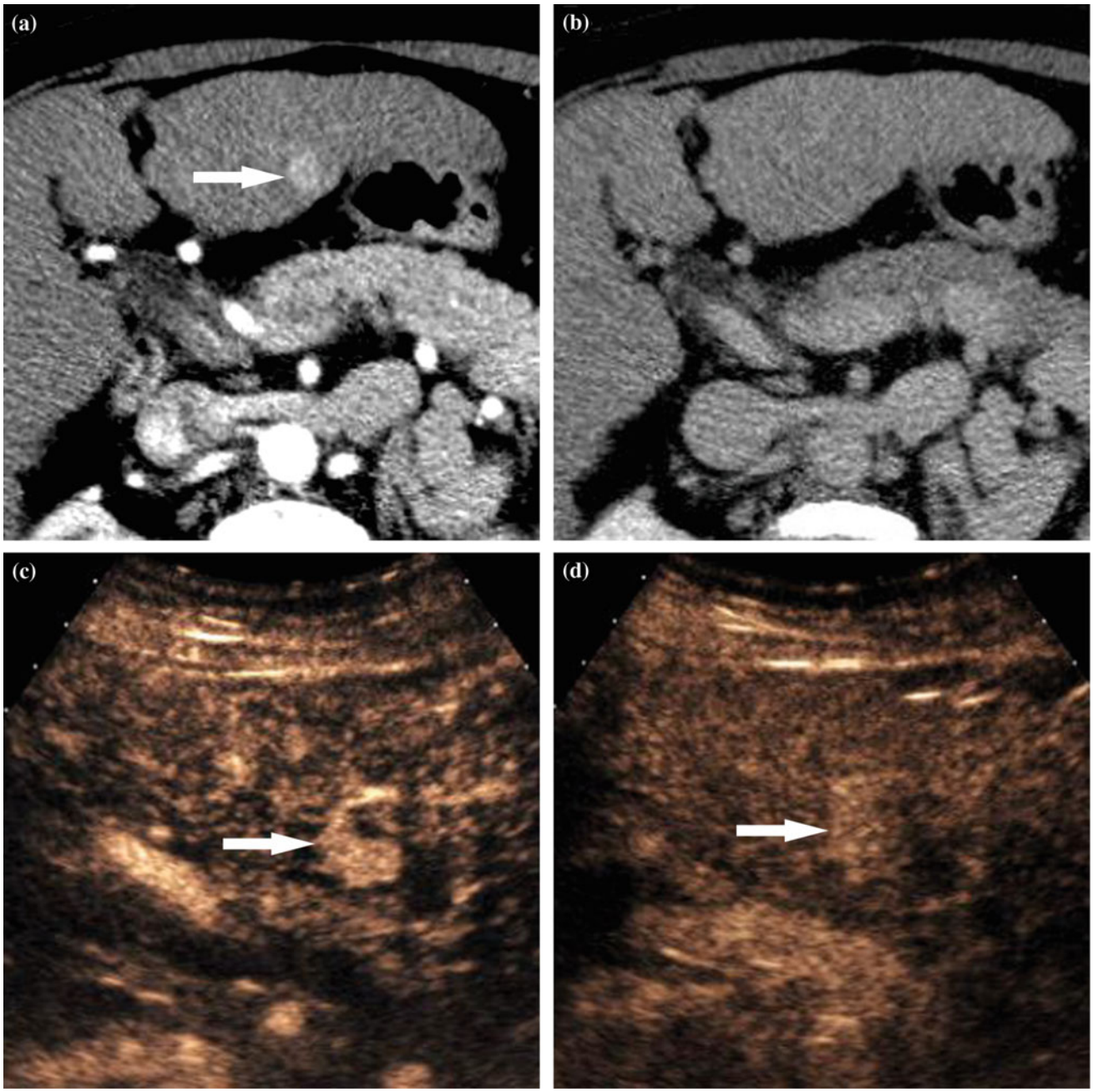

Fig. 24.9 HCC with no washout in a 53-year-old woman with hepatitis C. **a** CT scan in the arterial phase shows a hypervascular nodule (*arrow*) in the liver. **b** The nodule is not seen due to isoattenuation to the liver in the delayed phase. **c** CEUS scan in the arterial phase at 15 s shows a hypervascular nodule (*arrows*) in the liver. **d**. The nodule (*arrow*) remains hyperechoic on CEUS scan at 250 s. No washout is seen in either CT or CEUS

Diffuse hyperechogenicity on gray-scale US is a well-known typical finding of hemangioma. However, diffuse hyperechoic nodules are not specific for hemangioma in the setting of liver cirrhosis as DN or HCC with fatty metamorphosis can show similar findings (Fig. 24.13) and further evaluation should be performed [32]. Immediate performance of CEUS at the time of detection of such a nodule can achieve a diagnosis of hemangioma by demonstrating the characteristic enhancement pattern that includes peripheral nodular enhancement, gradual central fill-in, and sustained enhancement. This can avoid further imaging tests such as CT or MRI and reduces patient's additional hospital visits and anxiety as well as medical cost [7, 33]. CEUS is also useful to demonstrate the characteristic enhancement pattern in fast filling hemangiomas which often

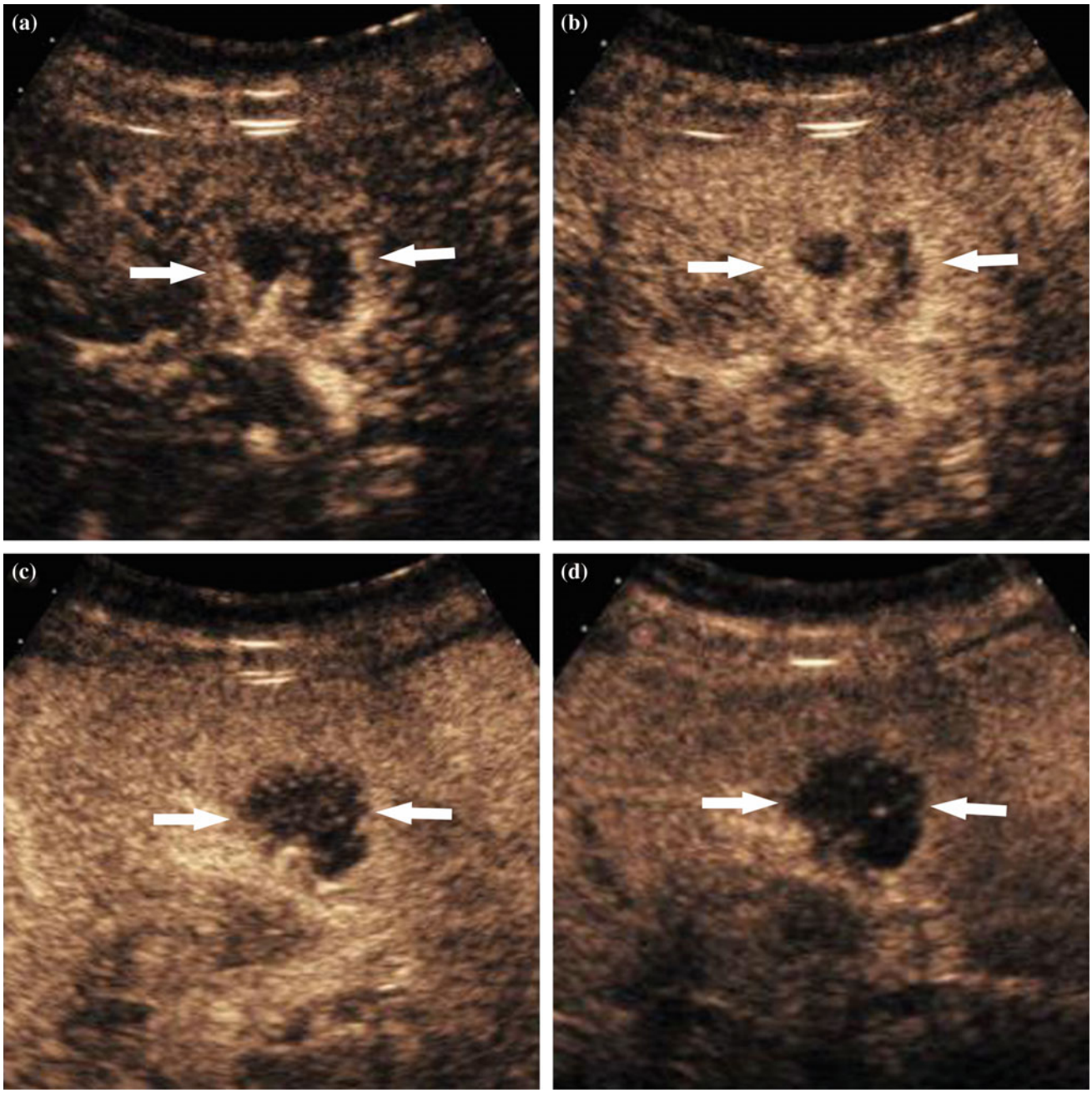

Fig. 24.10 Intrahepatic CC in an 80-year-old man with liver cirrhosis related to primary sclerosing cholangitis. **a** CEUS scan in the arterial phase at 14 s shows a mass (*arrows*) with rim-like hyperenhancement in the periphery. **b** The enhancing rim (*arrows*) becomes thicker and slightly more hyperechoic compared to adjacent normal liver at 21 s. **c**, **d** Washout (*arrows*) is seen at 40 s (**c**) and progresses to a punched-out lesion (*arrows*) at 3 min (**d**)

show a nonspecific homogeneous enhancement in the arterial phase of CT or MRI (Fig. 24.14). Slow filling hemangiomas, on the other hand, can be seen as nonspecific hypoattenuating masses on multiphasic CT scan. CEUS can diagnose a hemangioma in those cases by utilizing highly sensitive detection of contrast enhancement and prolonged observation (Fig. 24.15) [34].

Nontumorous arterioportal shunting is a common mimicker of malignancy in a cirrhotic liver and is frequently seen on multiphasic CT or MRI [35, 36]. It is typically wedge-shaped, peripherally located, and homogeneously hypervascular in the arterial phase. The lesion becomes isointense to the liver in the late phase and never shows washout. This potentially creates a pseudolesion as the

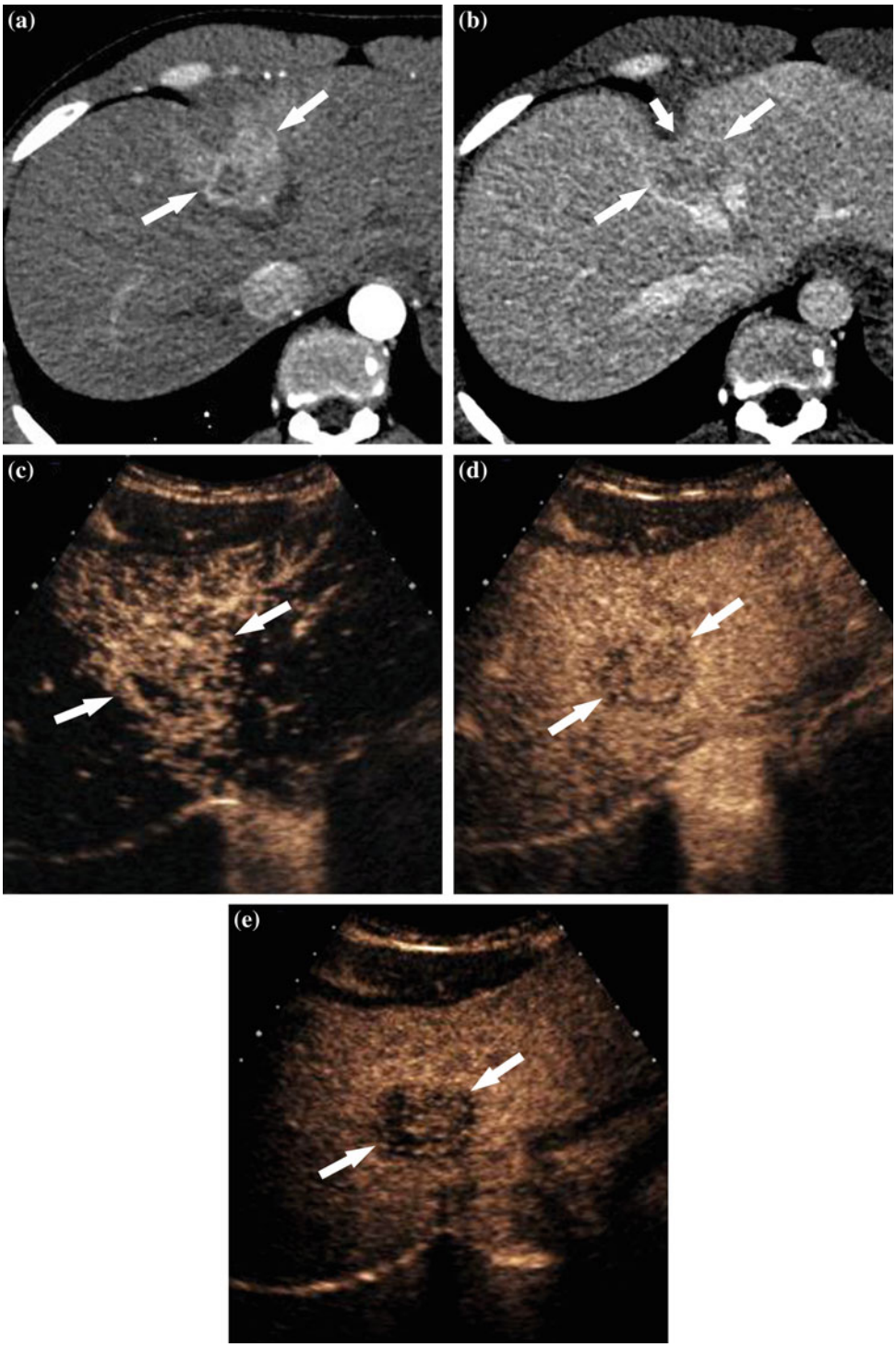

Fig. 24.11 Combined HCC and CC in a 36-year-old man with hepatitis B. **a** CT scan in the arterial phase shows a hypervascular mass (*arrows*) in the left lobe of the liver. **b** The mass (*arrows*) shows washout in the delayed phase. Note hepatic capsular retraction (*short arrow*) near the mass. **c** CEUS scan in the arterial phase at 10 s shows heterogeneous hypervascularity in the mass (*arrows*). **d** The mass (*arrows*) shows rapid washout at 40 s, which is unusual for HCC. **e** Washout progresses and the mass (*arrows*) is markedly hypoechoic at 180 s

differentiation of arterioportal shunting from HCC without washout is difficult. Nontumorous arterioportal shunting is not seen on gray-scale US as it is not a real parenchymal liver lesion. Therefore, CEUS is excellent to resolve this dilemma showing no abnormality in the presence of shunting. By comparison, if an HCC is present, ultrasound will show a nodule with appropriate CEUS characteristics [22].

24.4 Role of CEUS in HCC Diagnosis and Staging

Surveillance for HCC in high-risk patients is widely practiced particularly in endemic regions of hepatitis B and C, such as East Asia. Surveillance generally includes US at 6 month intervals. Further contrast-enhanced diagnostic imaging tests

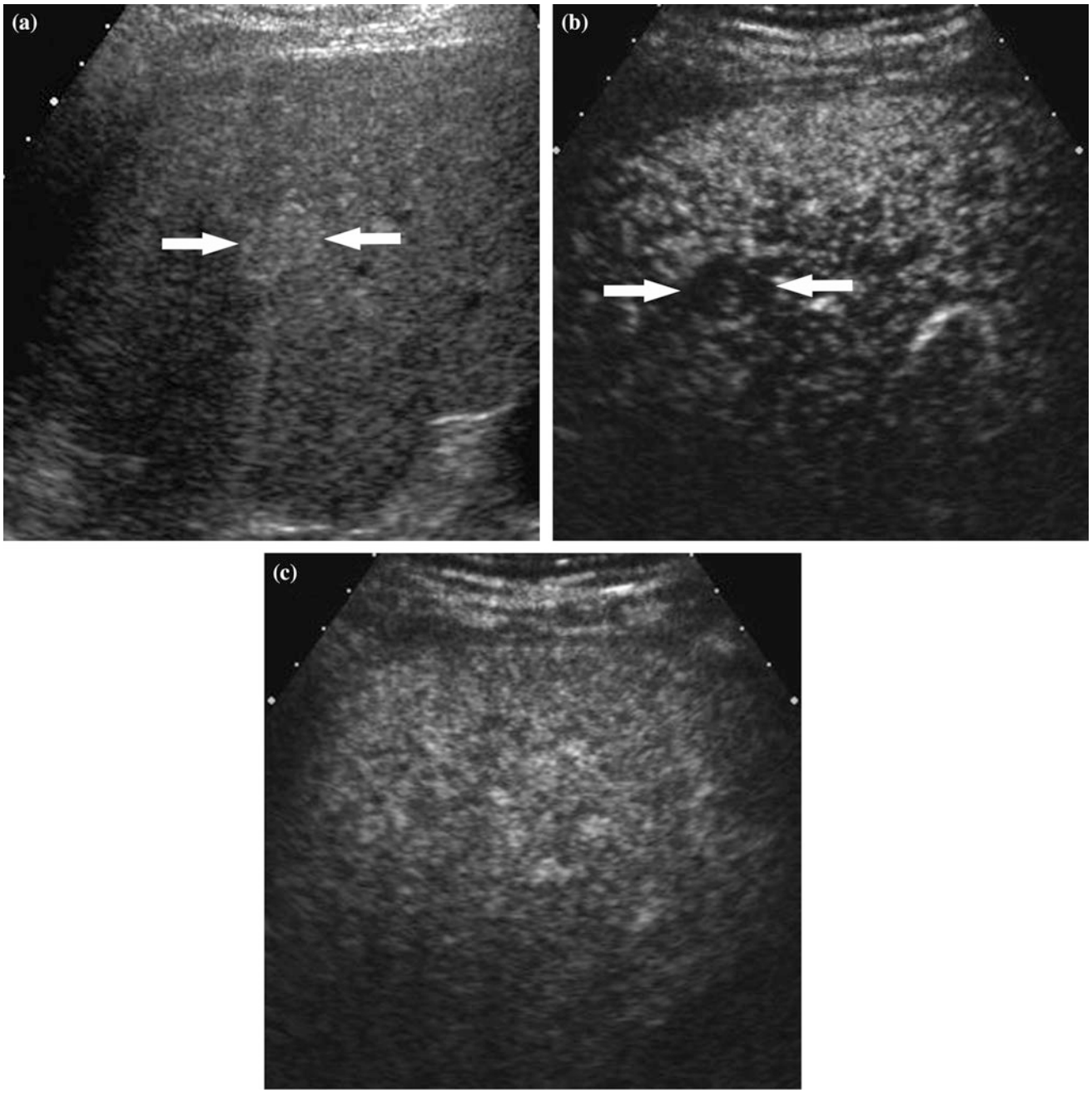

Fig. 24.12 Dysplastic nodule in a 39-year-old man with hepatitis B. **a** US scan shows a slightly hyperechoic nodule (*arrows*) in the liver. **b** CEUS scan in the arterial phase at 13 s shows decreased arterial-phase vascularity (hypovascular) within the nodule (*arrows*) relative to the liver. **c** The nodule is not seen due to isoechogenicity at 120 s

are performed when there is any new liver nodule 1 cm or larger found at surveillance US. The diagnosis of HCC can be made without biopsy when the nodules show typical findings on diagnostic imaging tests. Recent practice guidelines define a 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 [3–5].

There has been a controversy on the use of CEUS in international guidelines with exclusion in the most recent AASLD guidelines because of the claim that intrahepatic CC can mimic HCC with resultant misdiagnosis [3, 25]. However, subsequent rebuttal suggests that intrahepatic CC is relatively rare in liver cirrhosis and CEUS can depict typical findings of CC including arterial-phase rim enhancement (Fig. 24.10), rapid washout (<60 s), and/or a punched-out

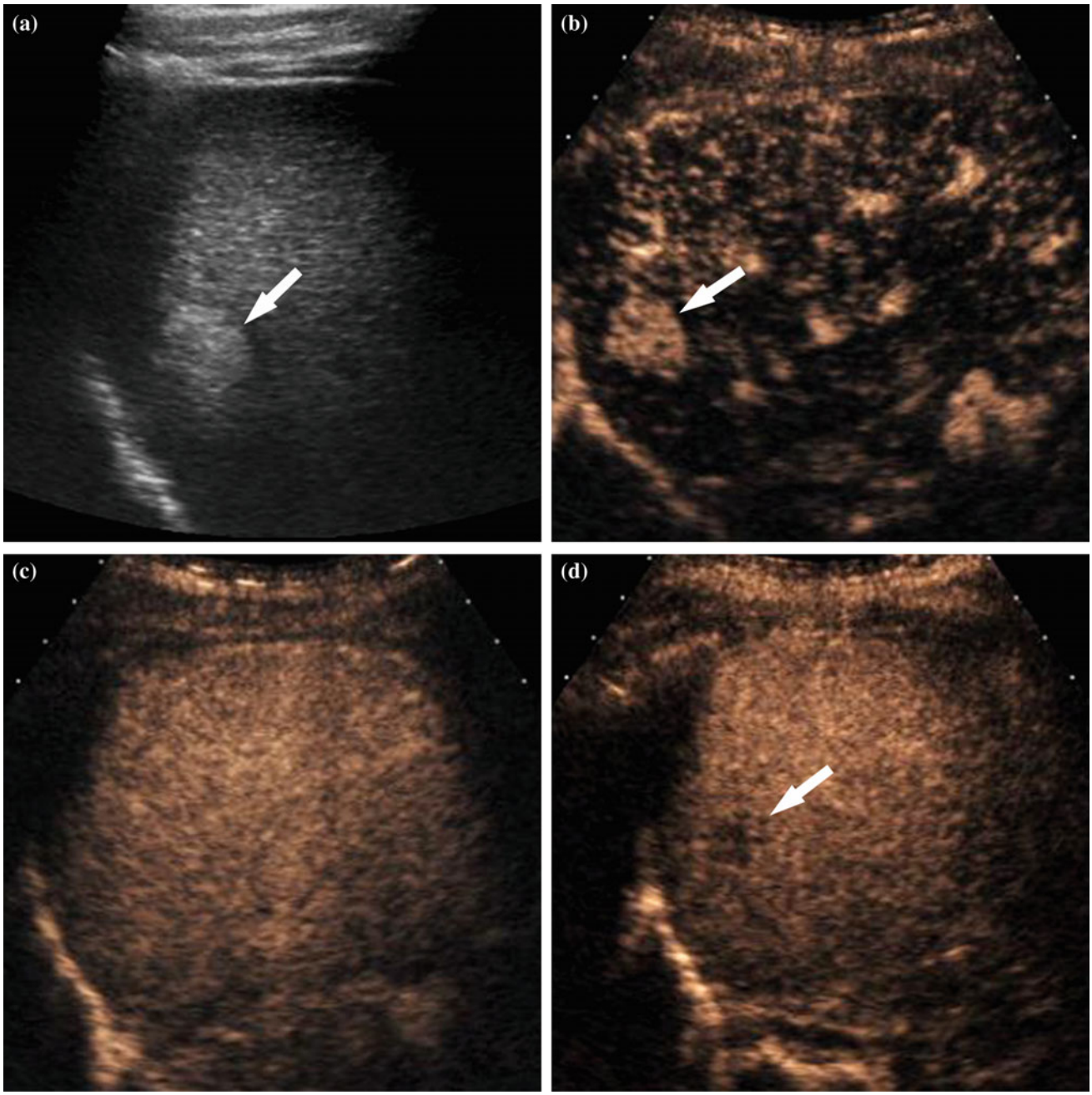

Fig. 24.13 HCC in a 75-year-old man with hepatitis B. **a** US scan shows a homogeneously hyperechoic nodule (*arrow*) in the liver, which mimics the gray-scale appearance of hemangioma. **b** CEUS scan in the arterial phase at 27 s shows homogeneous hypervascularity within the nodule (*arrow*). **c** The nodule is not seen due to isoechogenicity at 125 s. **d** The nodule (*arrow*) shows washout at 240 s, confirming the diagnosis of HCC

appearance in the late phase (Fig. 24.8) [20–22, 26, 37]. In fact, CEUS is still actively used as one of the diagnostic tests for HCC in other jurisdictions (for example, Italy, Japan, and Canada) and in large academic institutions where CEUS is available. CEUS is very well accepted by clinicians as it often plays a crucial role in diagnosing indeterminate nodules on CT or MRI and in diagnosing liver nodules in patients with renal failure (Fig. 24.16) [33]. Recently a CEUS working group has been formed in Liver Imaging Reporting and Data System (LI-RADS) by the American College of Radiology. LI-RADS aims to reduce imaging interpretation variability and errors to optimize diagnosis of HCC [38].

Multiphasic CT or MRI are proper staging techniques for HCC and should be performed once the diagnosis of HCC is made. There are occasional cases, however, where critical

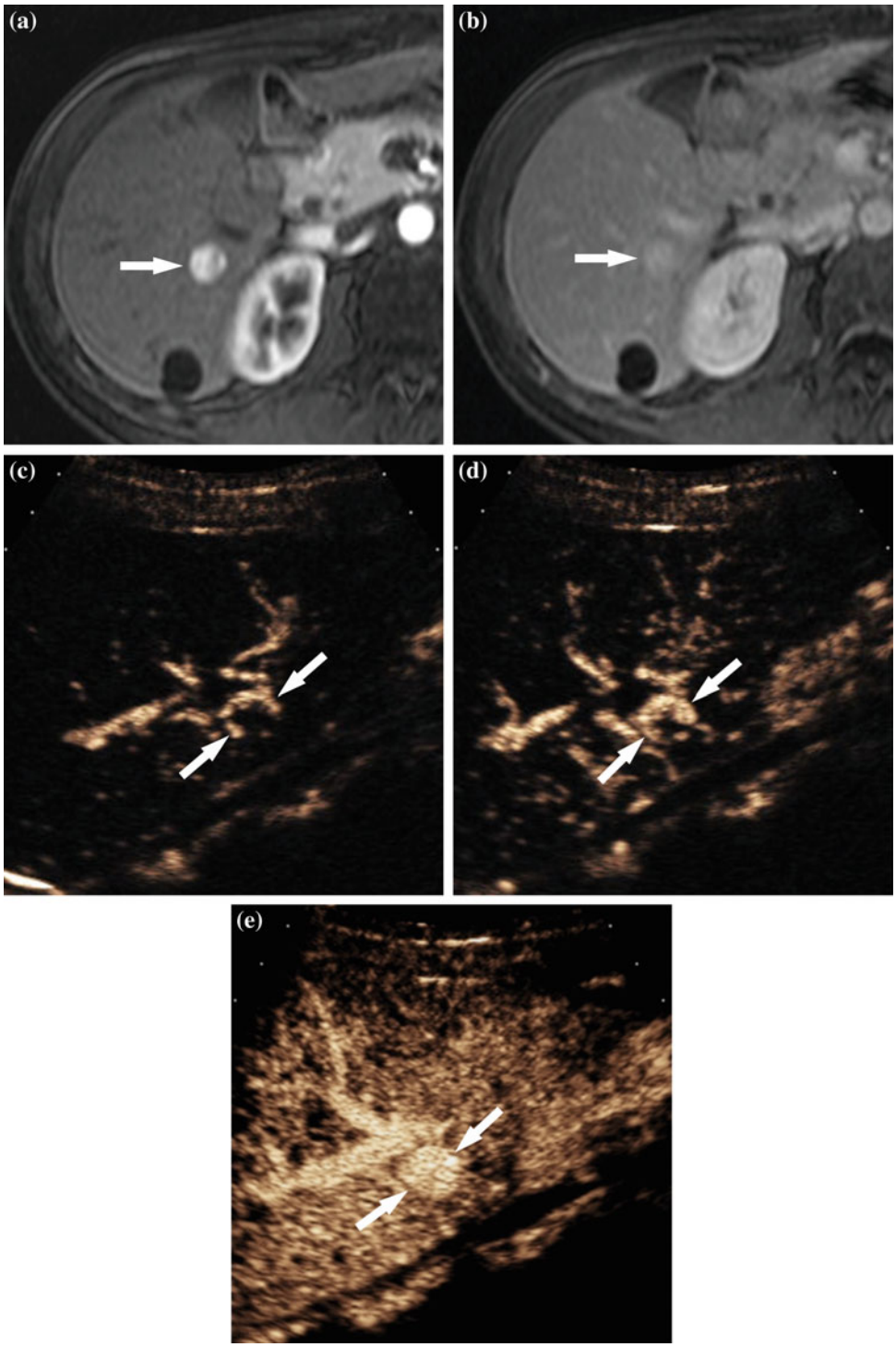

Fig. 24.14 Hemangioma in a 60-year-old woman with hepatitis B. **a** Contrast-enhanced T1-weighted MR scan in the arterial phase shows a slightly heterogeneous hyperenhancing nodule (*arrow*) in the liver. **b** The nodule (*arrow*) is homogenously hyperintense in the delayed phase. MR findings are indeterminate as the arterial-phase enhancement pattern is nonspecific and there is no washout. **c–e** CEUS scans at 9 (**b**), 10 (**c**), and 35 (**d**) seconds after injection of the contrast material show peripheral nodular enhancement with subsequent central fill-in in the nodule (*arrows*), which is diagnostic of hemangioma

staging information such as tumor thrombosis within the portal or hepatic vein is unclear on CT or MRI. The presence of malignant thrombus of portal or hepatic veins in patients with HCC is a critical determinant of tumor staging and prognosis as it directly influences treatment strategy [39, 40]. Bland thrombus can be found in 4.5–26 % of patients with chronic liver disease and up to 42 % of patients with HCC [41]. Moreover, malignant venous thrombus can occur in the absence of primary parenchymal HCC, either as an intravascular growth of this neoplasm [42] or after treatments such as ablation or chemoembolization, as a first indicator of recurrence.

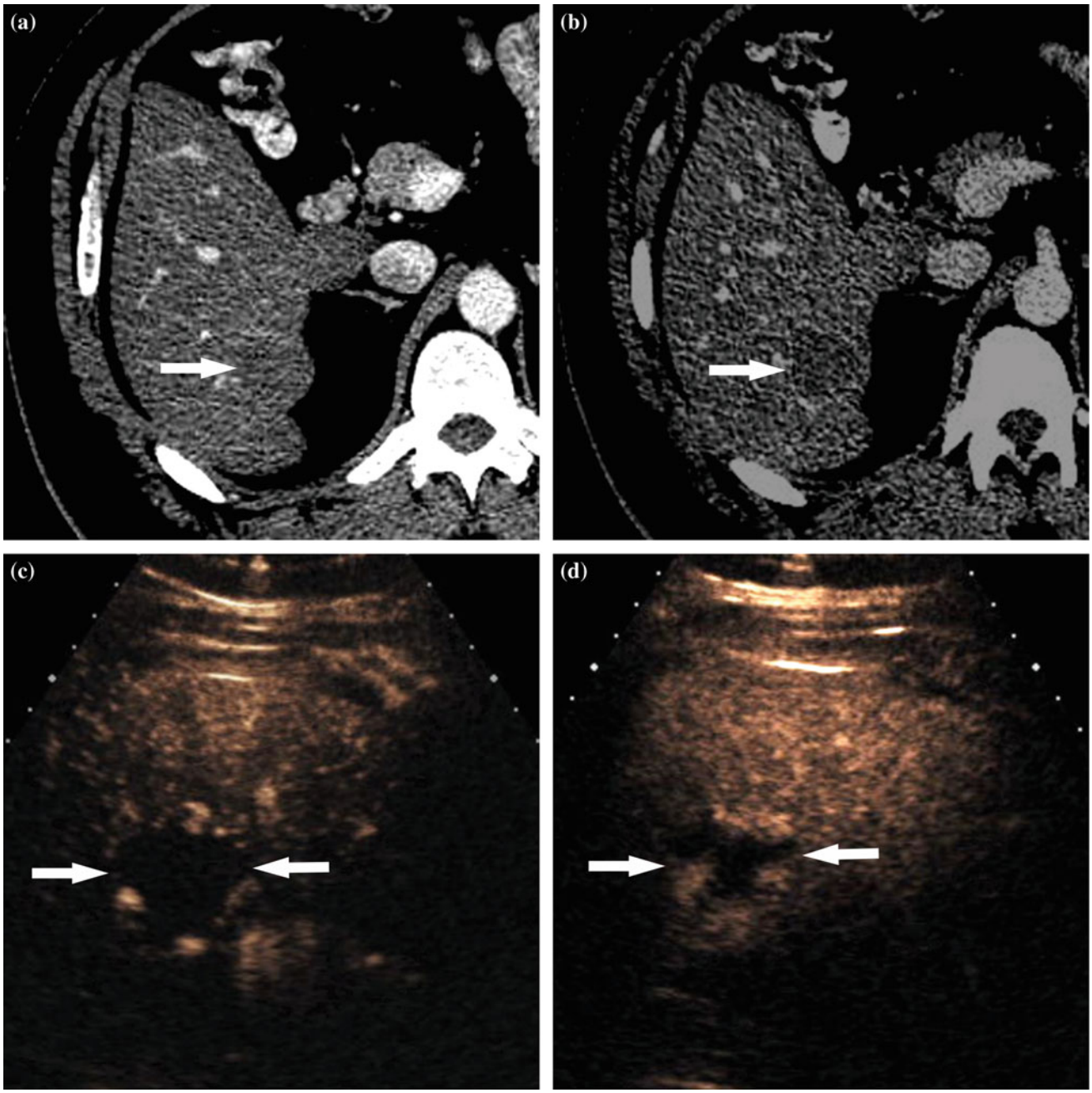

Fig. 24.15 Hemangioma in a 41-year-old woman with non-alcoholic steatohepatitis. **a** CT scan in the arterial phase shows a subtle hypoattenuating mass (*arrow*) in the liver. **b** The mass (*arrow*) is hypoattenuating in the delayed phase. CT findings are indeterminate. **c**, **d** CEUS scans at 15 (**c**) and 100 (**d**) seconds after injection of the contrast material show peripheral nodular enhancement with subsequent central fill-in in the nodule (*arrows*), which is diagnostic of hemangioma

CEUS is excellent in the differentiation of tumor thrombosis and benign thrombosis in the portal or hepatic veins. Tumor thrombi invariably show heterogeneous enhancement and linear, irregular feeding vessels after injection of the microbubbles in the arterial phase (Fig. 24.17), whereas benign thrombi are avascular (Fig. 24.18). In our study of 50 HCC patients with 38 malignant and 13 benign venous thrombosis, the area under the curve (AUC) at receiver operating characteristic (ROC) analysis was 0.947 and 0.958 by two independent blind readers. Demonstration of arterial flow within the thrombi is specific for malignant thrombosis; however, it is important to be aware that recanalized benign thrombosis may show enhancement in the portal venous phase [8].

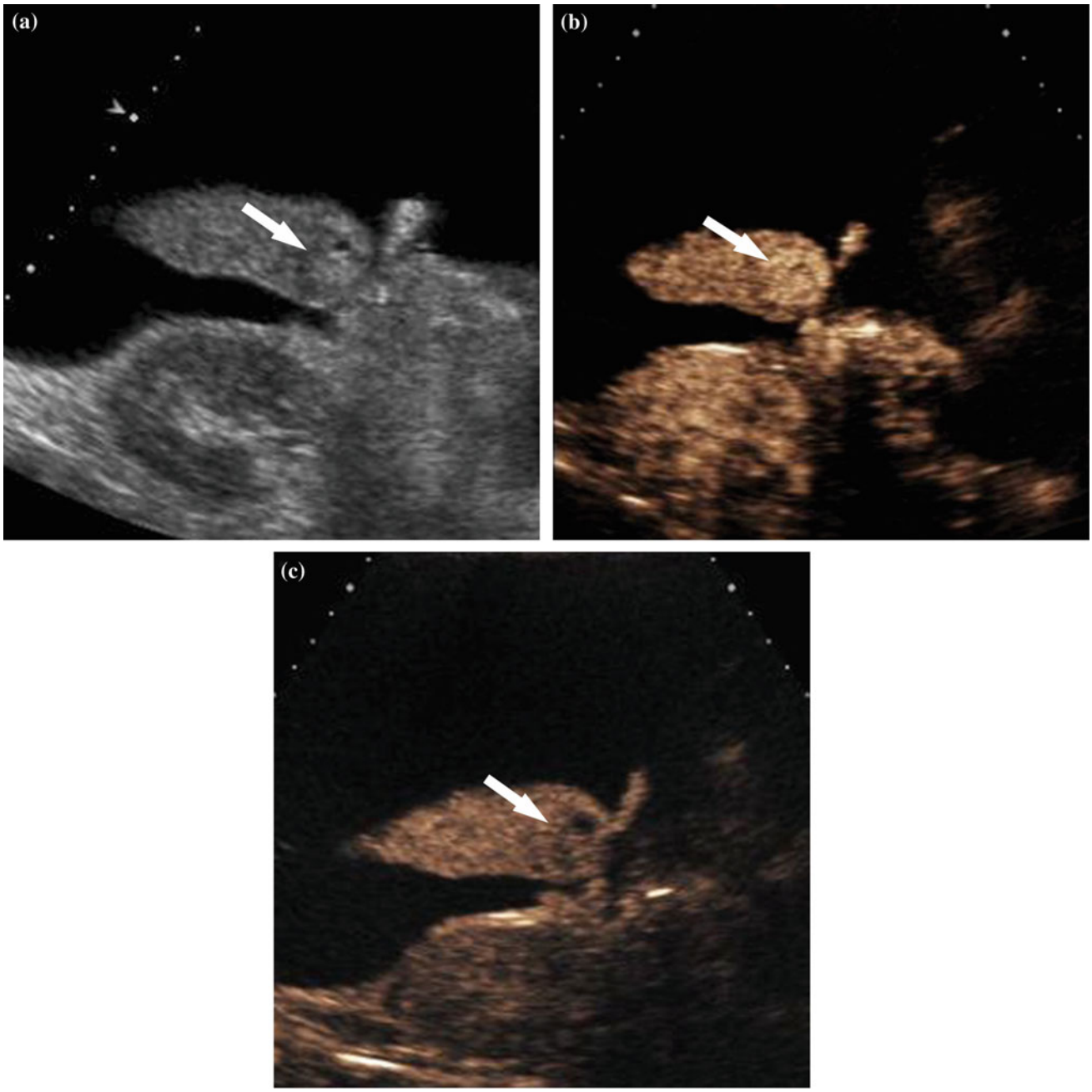

Fig. 24.16 HCC in a 52-year-old man with hepatitis C cirrhosis and renal failure. **a** US scan shows a slightly hypoechoic nodule (*arrow*) in the liver. Contrast-enhanced CT or MRI could not be performed due to renal failure. **b** CEUS scan in the arterial phase at 24 s shows homogeneous hypervascularity within the nodule (*arrow*). **c** The nodule (*arrow*) shows washout at 90 s, confirming the diagnosis of HCC

24.5 Post-treatment Monitoring of HCC

Local ablative therapy such as radiofrequency (RFA) or microwave ablation has become one of the main treatment modalities for patients with small HCC. RFA is also frequently performed as a bridge therapy for patients on the waiting list for liver transplantation. Real-time gray-scale US scan is most frequently used for the guidance of RFA procedures; however, there are uncommon cases with poor visibility on US scan. CEUS can be extremely helpful in these situations to localize the lesion by demonstrating the arterial-phase hypervascularity and washout. The use of a 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 [43]. A routine use of pre-procedure CEUS can reduce the number of

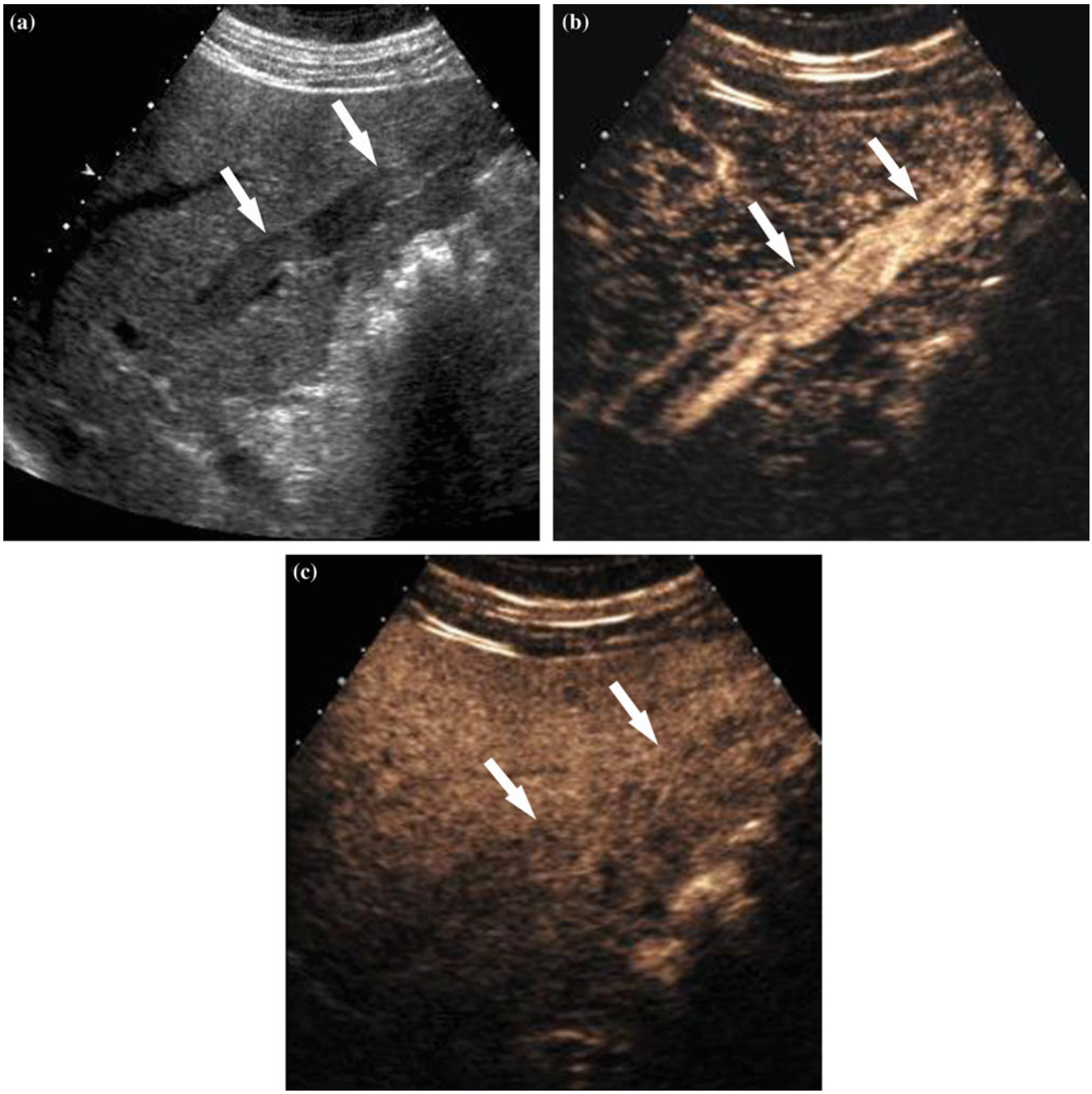

Fig. 24.17 Tumor thrombosis in the portal vein in a 70-year-old man with HCC and hepatitis C. **a** US scan shows a hypoechoic tubular lesion (*arrows*) in the liver, representing thrombosis within the portal vein. **b** CEUS scan in the arterial phase at 15 s shows strong, homogeneous enhancement within the portal venous thrombi (*arrows*). **c** There is mild washout (*arrows*) at 200 s, confirming the diagnosis of tumor thrombosis in the portal vein

incomplete or erroneous RFA significantly. Fusion imaging techniques that can coordinate the CEUS images with CT/MRI images are also helpful to localize difficult lesions and reduce the overall procedure time [44]. One of the unique advantages of CEUS in RFA is that the microbubbles can be repeatedly injected over short intervals as necessary. For example, CEUS can be performed just before the placement of the ablation needle and repeated after the needle placement to ensure its proper location. CEUS can be also performed after ablation to determine the completeness of the therapy. Repeat ablation can be immediately performed if there is any residual enhancing tumor [45, 46].

Multiphasic CT or MRI is typically performed in 1 month after ablative therapy for HCC in our institution. CT or MRI is an appropriate restaging modality as it provides information on the rest of the liver, vascular invasion, lymph nodes,

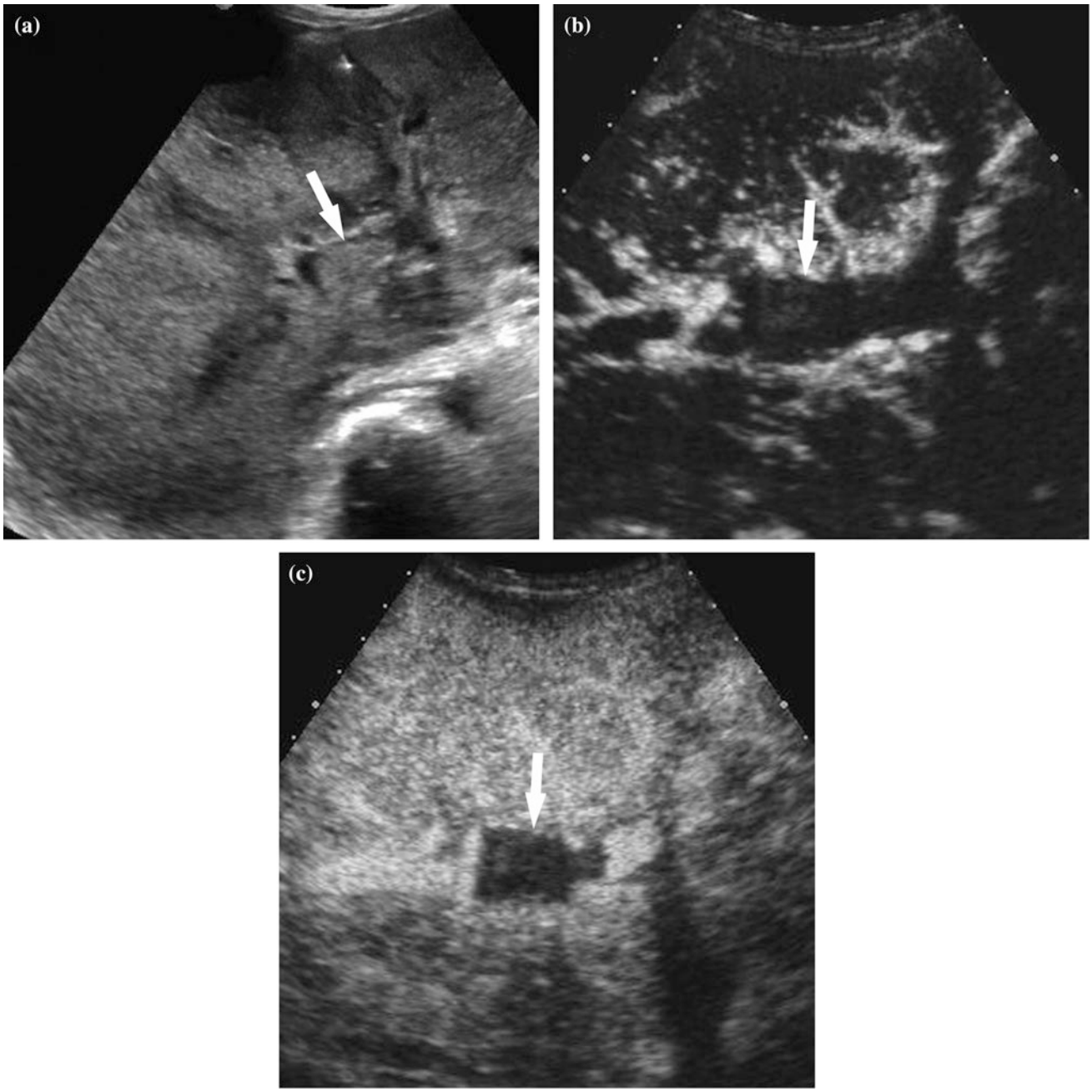

Fig. 24.18 Benign thrombosis in the portal vein in a 66-year-old man with hepatitis C and history of liver transplantation for HCC. **a** US scan shows a hyperechoic thrombosis (*arrow*) within the portal vein near the hilar hilum in the liver. **b**, **c** There is no contrast enhancement within the thrombus (*arrow*) on CEUS scans at 10 s (**b**) and 30 s (**c**), confirming the diagnosis of benign thrombosis in the portal vein

and any extrahepatic metastasis better than CEUS. CEUS is a useful alternative modality when the patient has renal failure with contraindication for CT or MRI contrast agent. One of the limitations of CEUS is that it is not possible to scan the whole liver in the arterial phase. Repeated sweepings through the entire liver in the late phase should be routinely performed to detect any unexpected recurrent HCC which is not detected on gray-scale US. While CT or MRI is useful for restaging HCC after ablation, there are occasional challenging cases with difficulty of indeterminate imaging findings. CEUS is an excellent problem-solving method in these cases [43]. Subsequent follow-up after therapy is variable and may include CEUS and/or MRI.

Hypervascular abnormalities adjacent to the ablation zone are common and can be residual HCC or benign perfusion abnormalities related to the ablation procedures [47]. These

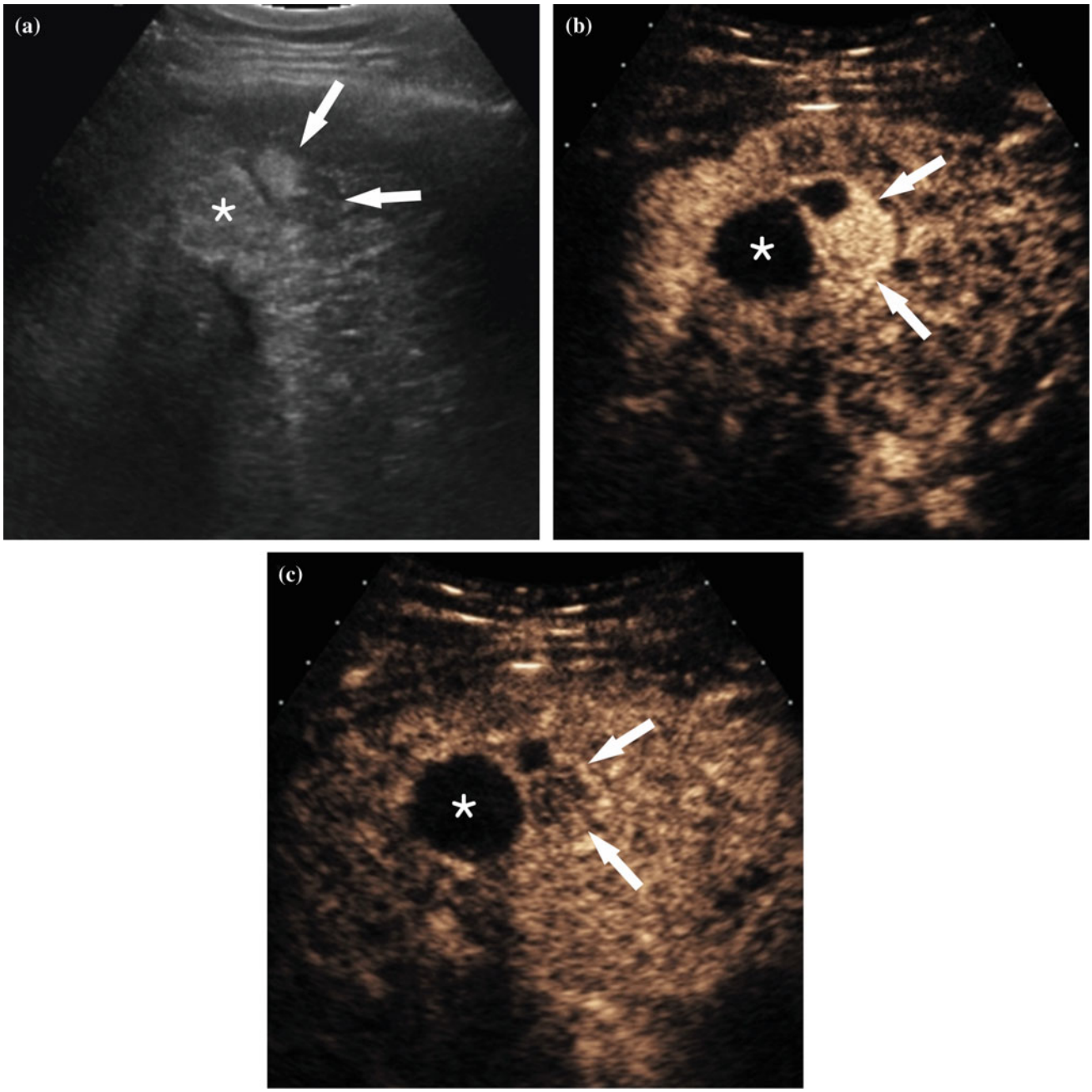

Fig. 24.19 Marginal tumor recurrence in a 63-year-old man who underwent radiofrequency ablation for HCC. **a** US scan shows a hyperechoic ablation zone (*asterisk*) and an adjacent mixed-echo lesion (*arrows*) in the liver. **b** CEUS scan in the arterial phase shows hypervascularity (*arrows*) within the mixed-echo lesion adjacent to the ablation zone (*asterisk*). **c** CEUS scan at 70 s shows washout (*arrows*), confirming the presence of marginal tumor recurrence adjacent to the ablation zone (*asterisk*). Repeat radiofrequency ablation was performed under CEUS guidance (not shown)

benign perfusion abnormalities adjacent to the ablation zone are frequently seen and may persist several months after the RFA procedure. The differentiation between benign perfusion abnormalities and recurrent HCC can be difficult when washout is not clearly seen on CT or MRI. Benign perfusion abnormalities are not seen on gray-scale US as they are not real lesions. Marginal recurrence of HCC is usually seen as a focal gray-scale abnormality adjacent to the ablation zone on unenhanced ultrasound. Subsequent CEUS shows hypervascularity followed by washout, confirming the presence of recurrent HCC and its exact location on grayscale US, which is extremely helpful for repeat ablation therapy (Fig. 24.19).

Recurrent HCC adjacent to the ablation zone is occasionally non-hypervascular on CT or MRI due to misiming of the

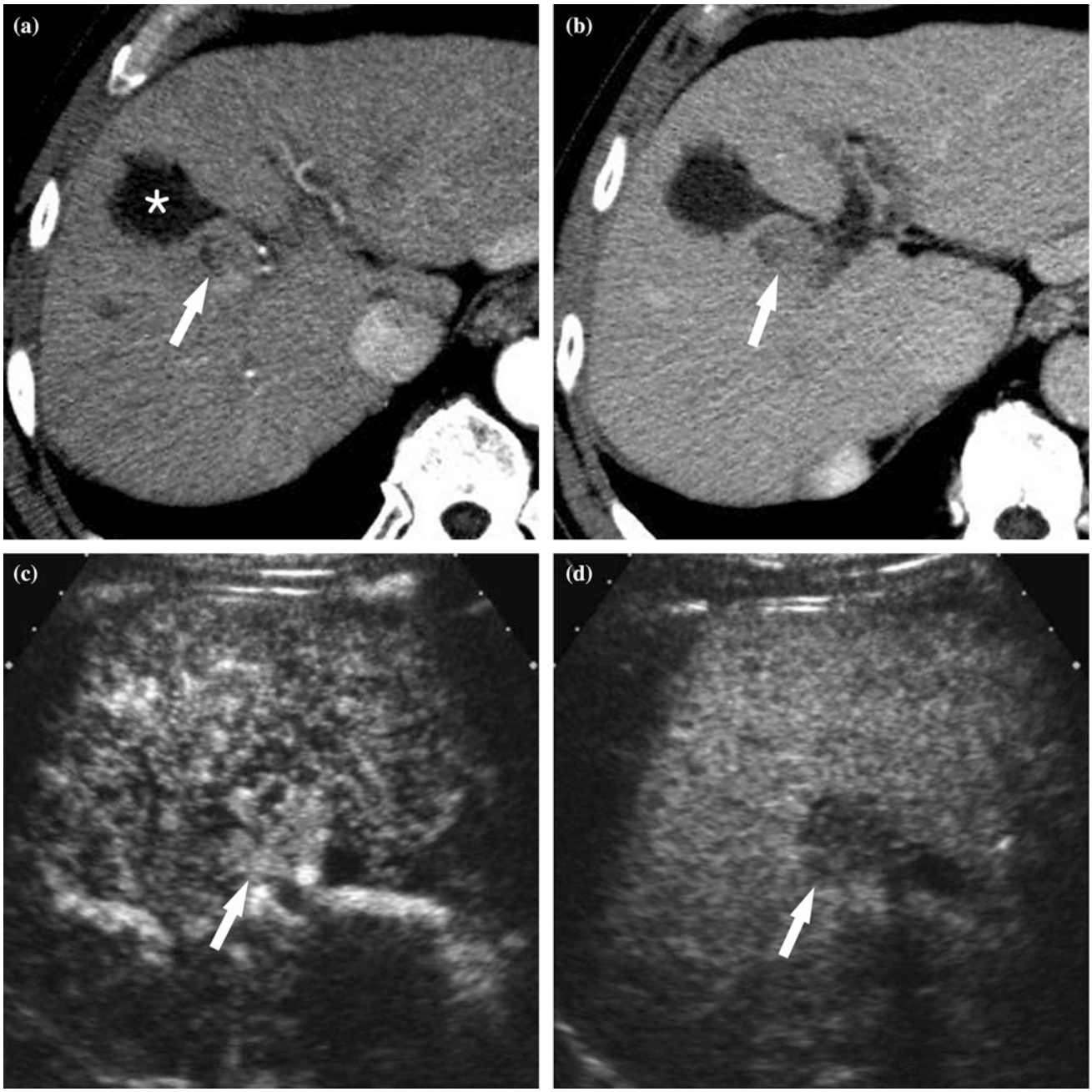

Fig. 24.20 Recurrent HCC in a 68-year-old man who underwent radiofrequency ablation for HCC. **a** CT scan in the arterial phase shows a subtle hypoattenuating lesion (*arrow*) medical to the ablation zone (*asterisk*). **b** The lesion (*arrow*) is hypoattenuating in the delayed phase. CT findings are indeterminate. **c** CEUS scans in the arterial phase at 12 s shows a focal hypervascular lesion (*arrow*). **D** The lesion (*arrow*) shows washout at 125 s, confirming the diagnosis of recurrent HCC

arterial phase. Subtle hypervascularity of recurrent HCC can also be obscured by adjacent perfusion changes. CEUS is useful to further assess hypoattenuating/hypointense abnormalities adjacent to the ablation zone on CT or MRI. CEUS can often show the presence of hypervascularity of the lesion, utilizing the advantage of real-time assessment of lesion perfusion (Fig. 24.20) [43].

24.6 Conclusion

CEUS is an excellent imaging technique with several unique advantages over CT or MRI for the imaging of nodules in a cirrhotic liver. These advantages in the arterial phase include the real-time depiction of specific features of benign hepatic nodules, resolution of arterioportal shunts, resolution of

absent enhancement on mistimed CT and MR scan, and sensitive demonstration of hypervascularity in HCC. Absence of washout of suspect HCC on CT or MR scan may also be resolved by CEUS. Therefore, CEUS can be effectively used as one of the diagnostic tests for HCC, differentiation between benign and malignant venous thrombosis, immediate diagnosis of hemangioma, and pre- or post-RFA evaluation for HCC. Added to this is the absence of nephrotoxicity of CEUS as well as the standard benefits of US including absence of ionizing radiation, and excellent patient compliance. Nonetheless, CEUS is operator-dependent and the performance of liver CEUS requires extensive hands-on experience.

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MRI for Detection and Evaluation of Hepatocellular Carcinoma

25

Anuj Patel, Neil Malhotra, Christopher G. Roth, Sandeep Deshmukh, and Donald G. Mitchell

Contents

25.1 Introduction

Patients with cirrhosis are at a risk for developing HCC. MRI and other radiologic imaging methods are often able to diagnose HCC in high-risk populations often before elevation in serum alpha-fetoprotein and other nonimaging signs [1, 2]. Additionally, the use of radiologic imaging for screening high-risk patients can expedite the treatment for suspicious lesions without confirmative biopsy [3]. Earlier detection allows for earlier treatment with the goal of detecting malignancy prior to extrahepatic spread.

This chapter considers the role of MRI in HCC detection. The chapter will focus mainly on current MRI protocols and imaging features used to evaluate liver lesions. Of particular importance, the current standardization of imaging reporting using Liver Imaging Reporting and Data Systems (LI-RADS) will be discussed.

25.2 Nonmalignant Focal Findings in Chronic Liver Disease

The cirrhotic liver has an abnormal shape and contour due to a combination of atrophy, hypertrophy, and scarring in different areas of the liver. Often times a focal finding in a cirrhotic liver may appear as a suspicious mass, but in fact just represents normal liver parenchyma surrounded by diseased tissue. This section discusses some of the common benign findings specific to chronic liver disease including confluent fibrosis, regenerative nodules, and dysplastic nodules. Other common benign liver findings such as cysts and hemangiomas will not be covered as they are not specific to cirrhosis. Malignant findings, primarily HCC, are discussed later in this chapter.

Confluent fibrosis develops in severely cirrhotic livers and contains very few if any hepatocytes. Confluent fibrosis is typically mildly T2 hyperintense relative to the background liver and can demonstrate delayed contrast enhancement, similar to fibrosis in other parts of the body. Confluent fibrosis is best distinguished from HCC by its shape, which is

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geographic rather than round. Another distinguishing feature is retraction of liver contour, rather than expansion [4].

As cirrhosis progresses the remaining normal liver parenchyma essentially consists of *regenerative nodules* surrounded by fibrous septae. Regenerative nodules are therefore benign consisting of normal functioning liver parenchyma. They demonstrate no abnormal enhancement and appear similar to normal background liver in all phases of dynamic contrast. Regenerative nodules can have varying appearance on T1-weighted imaging include T1 hyperintensity in cases of cholestatic nodules. Siderotic nodules represent a subtype of regenerative nodules with increased iron and therefore are T2 hypointense.

Dysplastic nodules represent an intermediary between regenerative nodules and HCC. They demonstrate dysplastic features such as nuclear atypia on histology but do not meet criteria for overt malignancy [5, 6]. Dysplastic nodules cannot always be definitively differentiated from HCC by imaging, especially in the case of high grade dysplasia. MRI features that favor dysplastic nodule over HCC include T1 hyperintensity, T2 hypointensity, and arterial phase hypointensity [7]. However, due to the poor differentiation of dysplastic nodules from HCC, the term is no longer routinely used.

Though some lesions are definitively benign based on imaging such as confluent fibrosis, cysts, or hemangiomas, other lesions can exist on a spectrum from benign to malignant as in the case of dysplastic nodules and HCC. For this reason, the LI-RADS was created to standardized the reporting of suspicion based on imaging features (Fig. 25.1). LI-RADS is more extensively discussed throughout this chapter.

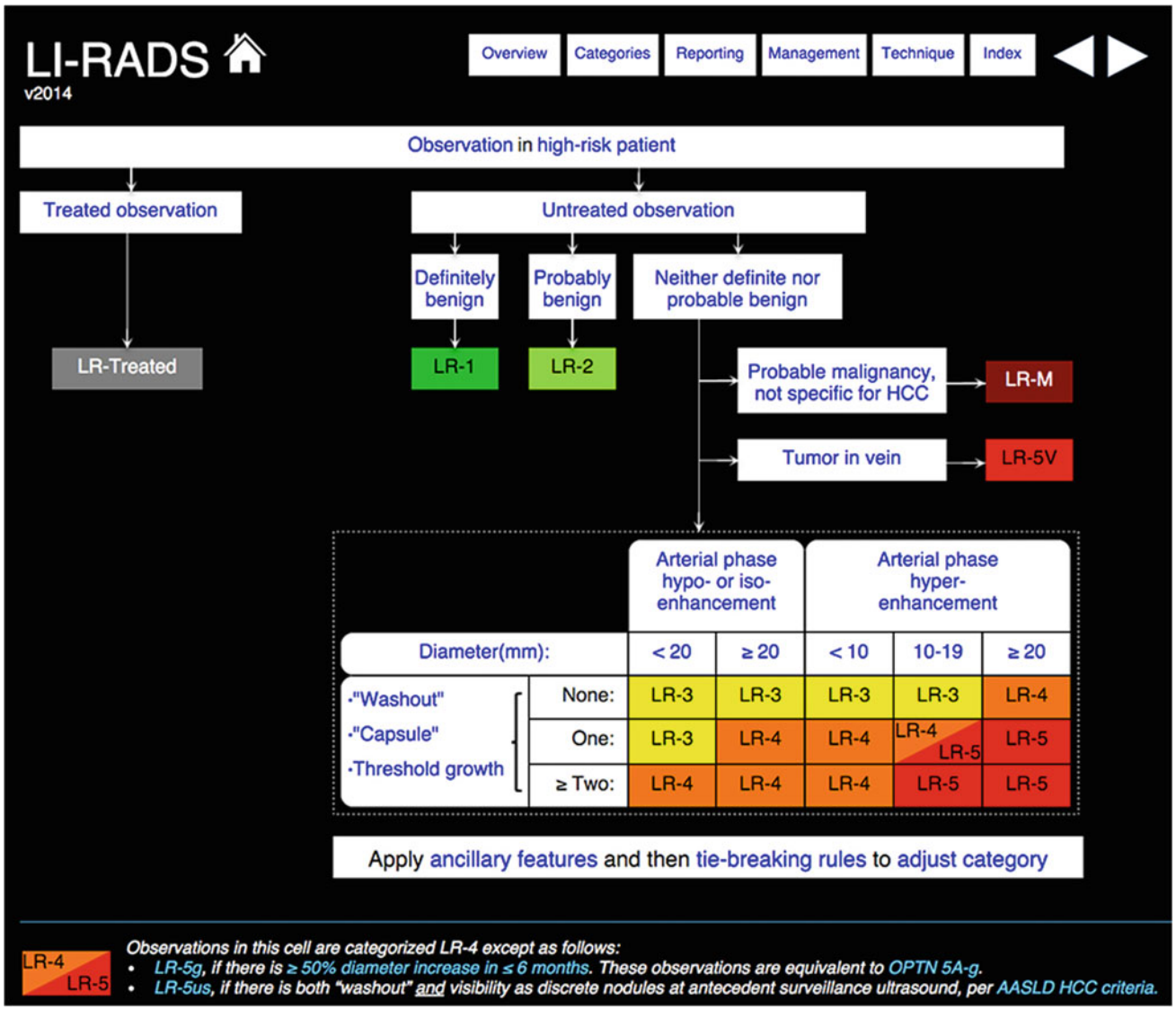

Fig. 25.1 LI-RADS algorithm version 2014. This algorithm depicts the decision-making pathway when reporting suspicion of hepatocellular carcinoma for liver observations

25.3 HCC MR Imaging Guidelines

25.3.1 Background

Advancements in treatment for HCC now include a variety of locoregional options in addition to surgery and liver transplant. Varying methods of imaging utilization are advocated based on different organizations including the American Association for the Study of Liver Diseases (AASLD), European Association for the Study of the Liver (EASL), Organ Procurement and Transplantation Network (OPTN), as well as others. The AASLD criteria have been prospectively verified and categorize imaging diagnoses as negative, indeterminate, or positive [3, 8–12].

Guidelines from the AASLD, EASL, and OPTN refer to imaging features of HCC including "arterial phase hyperenhancement" and "washout" without consensus from the groups regarding the exact definitions of these terms. The American College of Radiology gathered a panel of radiologists in 2008 to standardize criteria for the imaging diagnosis of HCC. This panel defined terminology and created a diagnostic imaging algorithm. LI-RADS was created by this panel in 2011 and has since been updated by radiologists, surgeons, hepatologists, and pathologists. The following information regarding this standardized system is based on LI-RADS v2014, which is fundamental to the current understanding of noninvasive imaging approaches to the diagnosis of HCC with emphasis on MRI in this particular chapter. Of particular note is the current lack of prospective validation of LI-RADS unlike that of AASLD guidelines.

LI-RADS applies only to patients with high risk of HCC which mostly includes patients with chronic liver disease or Hepatitis B. Definitely benign observations are categorized as LR-1, which is similar to the AASLD negative category. The AASLD category of indeterminate was expanded to three tiers in LI-RADS including probably benign (LR-2), intermediate (LR-3), and probably HCC (LR-4). Those observations that are definite HCC are categorized as LR-5, which is similar to the AASLD positive category. LIRADS includes an LR-M categorization for observations that are probably malignant but not specific to HCC, LR-5V when definite tumor is identified in a vein, and LR-Treated for lesions with previous locoregional treatment.

Category LR-1 and LR-2 have very broad overlap and commonly includes cysts, hemangiomas, vascular anomalies, perfusion alterations, confluent fibrosis, and focal scar. LR-2 is used for observations that may have slightly atypical features but are likely to represent one of these benign diagnoses. Additionally, LR-2 also includes regenerative nodules without suspicious major or ancillary imaging features, which are further described below [13].

The AASLD diagnosis of indeterminate leaves a broad range of uncertainty and can complicate the decision-making regarding imaging follow up, biopsy, or treatment recommendations when a lesion may be of low suspicion but not definitely benign. The expansion of the AASLD's indeterminate diagnosis to three tiers in LI-RADS (LR-2, LR-3, and LR-4) aids in relaying the radiologist's suspicion of an observation that is neither definitely benign nor definitely HCC. LI-RADS does not make category-based recommendations for imaging follow up, biopsy, or treatment. Rather, the clinical and diagnostic management of each patient is dependent on imaging evaluation combined with clinical status and available resources on an individual patient basis.

25.3.2 MRI Protocol

The minimum sequences recommended by both the OPTN and LI-RADS are in-phase/opposed-phase imaging, T2-weighted imaging, and T1-weighted gradient echo sequences with dynamic contrast enhancement including a precontrast sequence [13, 14]. LI-RADS further suggests but does not require diffusion weighted imaging, post-processing dynamic contrast subtraction, and mulitplanar acquisitions. The utility of these minimum required MRI sequences as well as the suggested technique of diffusion weighted imaging is described in this section.

25.3.2.1 In-Phase/Opposed-Phase Images

The detection of iron or microscopic/intralesional fat can be important in the evaluation of a focal liver lesion and is further described later in this chapter regarding ancillary imaging findings of HCC [15]. In-phase/Opposed-phase imaging utilizes T1-weighted dual echo gradients to exploit the miniscule difference in the precession frequency of hydrogen protons in water and fat in MRI. The two TEs (time to echo) are chosen such that signal from water and fat are at one point aligned (in-phase) and at the other time point are 180° apart (opposed-phase).

A drop in signal intensity in the opposed-phase compared to in-phase images is seen when fat and water are within the same imaging voxel. This is seen in hepatic steatosis or in primary hepatocyte containing liver lesions including HCC. A drop in signal intensity in the in-phase compared to the opposed-phase imaging is due to T2* dephasing as long as the TE of the in-phase image is longer than that of the opposed-phase imaging. This dephasing can be seen with susceptibility from metal artifacts or from increased iron content in states of iron overload.

25.3.2.2 T2-Weighted Images

T2-weighted images are commonly acquired utilizing moderate and heavy weighting. Moderately T2-weighted images are useful for showing both benign and malignant liver lesions with free or bound water. Lesion conspicuity is increased with fat suppression to null the inherent signal from fat.

Heavy T2-weighting is useful for showing lesions predominantly composed of free water such as benign hepatic cysts. Lesions comprised of free water have high signal intensity on T2-weighted images due to the long T2 of free water. Tumors as well as other benign lesions often show moderate T2 hyperintensity that is less prominent or not seen on heavily T2-weighted images due to the relatively lower T2 of bound water compared to free water within these lesions. Consequently, HCC demonstrates a mild or moderate T2 hyperintensity that may be visualized on moderately T2-weighted images. Of particular note is the infiltrative type of HCC, which is sometimes most conspicuous on moderately T2-weighted images even compared to dynamic post-contrast imaging.

25.3.2.3 Dynamic Contrast Images

Dynamic contrast imaging using extracellular fluid gadolinium-based agents is the workhorse in detection of HCC with MRI with the timing of each phase in this multiphase technique is important for optimizing the detection of HCC. A minimum of four phases of contrast should be obtained including unenhanced, late arterial, portal venous/blood pool (~20 s after arterial phase), and delayed (~3–5 min). The late arterial phase is when the arteries and portal venous system are both enhanced without hepatic vein enhancement.

HCC is fed predominantly from an arterial supply, whereas the normal liver parenchyma is fed from a predominately portal venous supply. This difference is exploited with dynamic multiphase contrast imaging because HCC will typically be high signal intensity relative to background liver in the late arterial phase but low signal relative to background liver in the portal venous phase [16].

Gadolinium-based contrast agents with partial hepatobiliary excretion such as gadoxetate disodium and gadobenate dimeglumine are newer contrast agents that are now becoming more commonly available. Approximately 20 min after contrast injection, the normal liver parenchyma will demonstrate uniform T1 hyperintensity due to the T1 shortening effects of the gadolinium-based contrast agent uptake by anion-transporting peptides in hepatocytes. This time period is referred to as the hepatobiliary phase. Signal intensity in the hepatobiliary phase can be used to determine the presence of functioning hepatocytes within observations in the liver. HCC and other malignancies typically do not containing functioning hepatocytes and will appear hypointense to normal liver parenchyma in the hepatobiliary phase.

25.3.2.4 Diffusion Weighted Imaging

LI-RADS suggests, but does not require, diffusion weighted imaging. Contrast in diffusion weighted imaging is dependent on differences in microscopic water motion. Malignant tumors tend to be highly cellular compared to benign lesions and will consequently demonstrate restricted diffusion, opposed to free bulk water motion.

25.4 HCC Imaging Features and Reporting Guidelines

25.4.1 Major Imaging Features

LI-RADS categorization of LR-3, LR-4, and LR-5 is based on the number of major features that are present as seen in Fig. 25.1. The major features include *arterial phase hyperenhancement*, *size*, *washout appearance*, *capsule appearance*, and *threshold growth*. Arterial phase hyperenhancement requires signal intensity greater than liver as well as the parenchyma surrounding the observation (Fig. 25.2d). Size is measured as the diameter of the observation in the single longest dimension. Washout appearance is the relative decreased signal intensity of the observation compared to background liver (Fig. 25.2e). Note that this is a relative feature and not based on quantitative analysis. Capsule appearance refers to portal venous or delayed phase enhancement around the periphery of an observation (Fig. 25.2f). This does not necessarily correspond to a capsule or pseudocapsule seen on histopathology. Threshold growth is defined as greater than 50 % growth in less than 6 months or greater that 100 % growth in greater than 6 months. Also, threshold growth is considered positive for any new observation greater than 10 mm. LR-5 (definitely HCC) can only be assigned to observations with arterial phase hyperenhancement as seen on the algorithm in Fig. 25.1 [13].

25.4.2 Ancillary Imaging Features

Ancillary features are features that can favor benignity or malignancy and may be used secondarily to alter the final category assigned to an observation (Tables 25.1 and 25.2). However, ancillary features cannot be used to upgrade a lesion to LR-5. This maintains the high specificity for HCC in LR-5 to remain in agreement with OPTN. Also, it is important to note that the ancillary features that favor malignancy are not necessarily specific for HCC but may represent any malignancy [13].

Those features which are more specific for HCC include *nodule-in-nodule appearance*, *mosaic architecture*, and *intralesional fat.* Nodule-in-nodule appearance describes a T2 hyperintense nodule within an T2 hypointense nodule. This

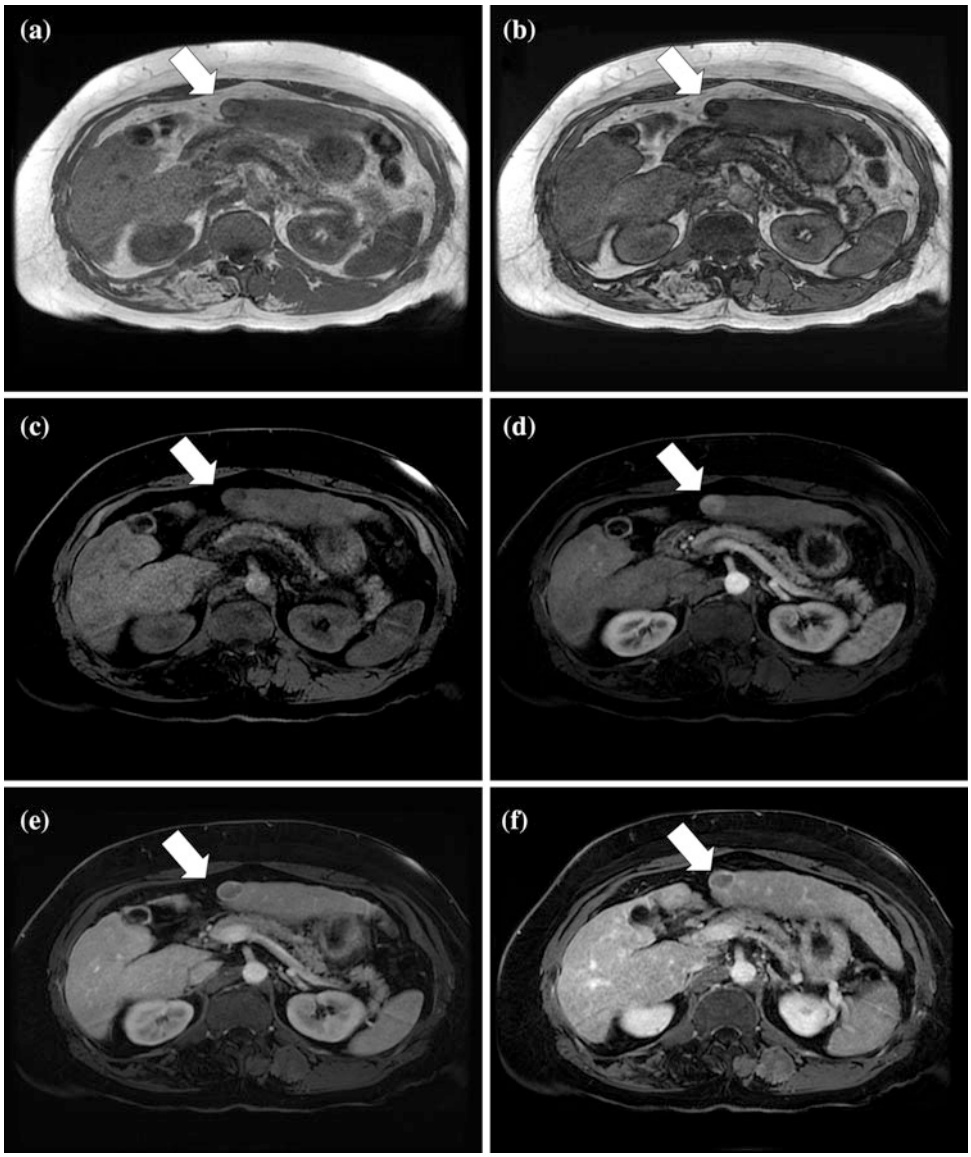

Fig. 25.2 Typical MRI features of HCC. **a** In-phase image shows HCC as nearly isointense to background liver. **b** Out-of-phase image shows HCC signal drop out relative to the In-phase image due to microscopic lipid. **c** Fat-suppressed T1-weighted precontrast image shows HCC as slightly hypointense to background liver. **d** Fat-suppressed T1-weighted early arterial phase contrast shows HCC as hyperenhancing relative to background liver. **e** Fat-suppressed T1-weighted late arterial phase image shows HCC as hypointense relative to background liver consistent with washout appearance. **f** Fat-suppressed T1-weighted delayed phase image shows HCC with delayed enhancing capsule appearance

Table 25.1 Benign ancillary features

Benign ancillary features
Homogenous marked T2 hyperintensity or hypointensity
Follows dynamic blood pool enhancement
Decreased size or stability over 2 years
Hepatobiliary phase isointensity

Ancillary features favoring benignity are used to decrease the level of suspicion for malignancy by adjusting the LI-RADS category to a lower level

Table 25.2 Malignant ancillary features

Malignant ancillary features
Mosaic architecture*
Nodule-in-nodule appearance on T2 weighted imaging*
Intralesional fat*
Mild/moderate T2 hyperintensity
Lesional fat/iron sparing
Blood products
Hepatobiliary phase hypointensity

Ancillary features favoring malignancy can upgrade the level of suspicion for malignancy up to LR-4, but not LR-5. Those labeled with an asterisk (*) are considered more specific for hepatocellular carcinoma over other malignancy

characteristic is a relatively specific sign of the malignant degeneration of a dysplastic nodule [17]. It is important to note that the nodule-in-nodule appearance is only described on T2-weighted imaging and not in reference to contrast

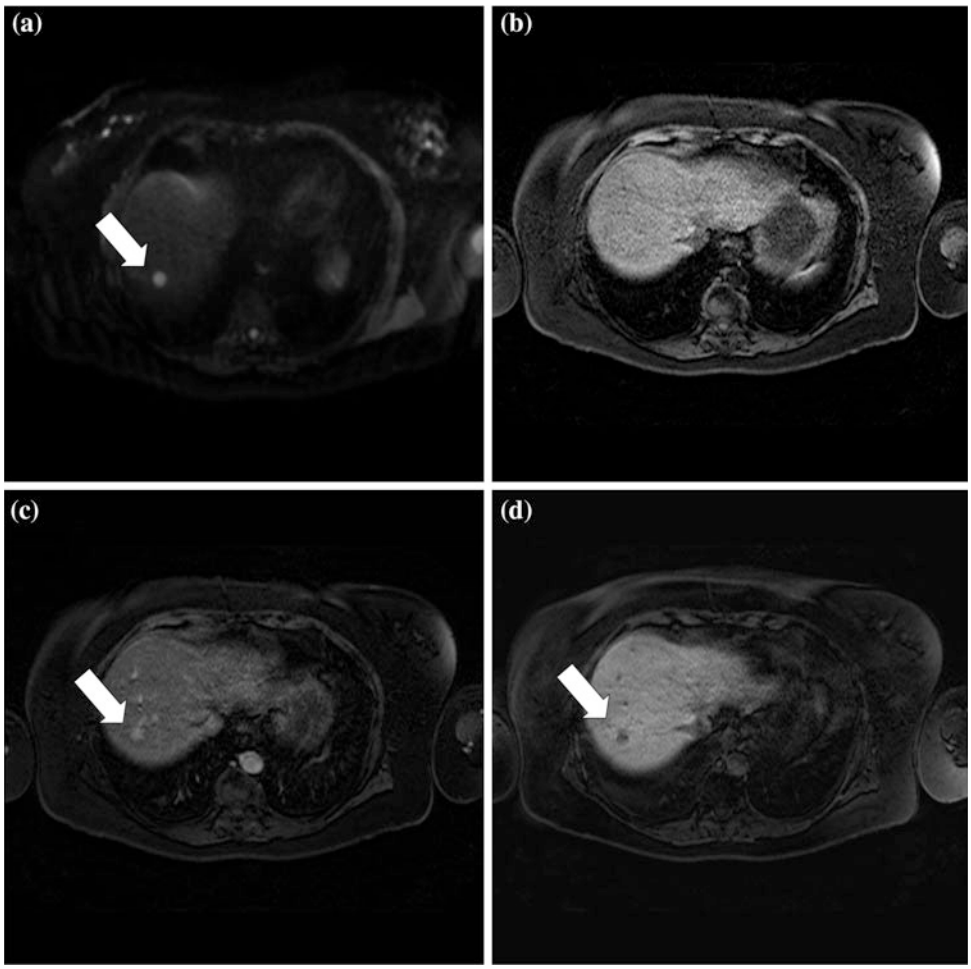

Fig. 25.3 Nonspecific MRI features that suggest malignancy but are not specific to hepatocellular carcinoma in this patient with hepatic metastatic melanoma. **a** Diffusion weighted image shows HCC as high intensity due to diffusion restriction. **b** Fat-suppressed T1-weighted precontrast image shows no detectable abnormality. **c** Fat-suppressed T1-weighted early arterial phase image using hepatobiliary-specific contrast (gadoxetate disodium) shows arterial hyperenhancement of the metastatic mass. **d** Fat-suppressed T1-weighted hepatobiliary phase image using hepatobiliary-specific contrast (gadoxetate disodium) shows hypointensity of the metastatic mass relative to normal liver parenchyma indicating lack properly functioning hepatocytes within the mass

enhancement characteristics. Mosaic architecture, however, describes either multiple nodules with varying levels of enhancement or internal enhancing septae. Intralesional fat is best visualized as a drop in signal intensity on opposed-phase compared to in-phase sequences (Fig. 25.3a, b) [13].

Diffusion restriction is an ancillary finding that favors malignancy in LIRADS but is not specific for HCC (Fig. 25.3a). HCC diagnosis accuracy is improved with combination of dynamic contrast MRI with diffusion weighted imaging [18–20]. However, diffusion weighted imaging is not considered a major imaging feature due to its lack of sensitivity and specificity [21]. Diffusion weighted imaging is also not routinely available on all MRI systems, and therefore, is not currently part of the required MRI protocol by LIRADS as outlined above [13].

Hepatobiliary phase hypointensity using gadolinium-based contrast agents with partial hepatobiliary excretion is another nonspecific ancillary feature that favors malignancy (Fig. 25.3d) [1, 13]. HCC, cholangiocarcinoma, and metastatic masses will typically not uptake the hepatobiliary-specific contrast due to lack of expression of the anion-transporting peptide and will consequently be hypointense compared to the background liver. However, 10–15 % of HCC can uptake the contrast and appear isointense or hyperintense in the hepatobiliary phase. Advanced cirrhosis can also cause heterogeneous signal intensity in the hepatobiliary phase which decreases sensitivity for HCC detection [22].

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Computed Tomography of HCC

26

Francesco Agnello and Giuseppe Brancatelli

Contents

26.1 Introduction

Contrast-enhanced computed tomography (CT) is the primary imaging technique for the study of the cirrhotic liver and hepatocellular carcinoma (HCC). CT allows an accurate, noninvasive diagnosis of HCC nodules, and can differentiate HCC nodules from other benign lesions such as hemangioma, and malignant lesions such as cholangiocarcinoma and metastasis. Pretreatment CT influences the selection of HCC treatment option by defining HCC number, size, location, and relationship with surrounding structures, along with the evaluation of hepatic vascular anatomy and patency [1]. For instance, HCC multifocality and invasion of major vessels can contraindicate surgical treatment, while substantial portal vein thrombosis precludes TACE because of the increased risk of liver failure due to hepatic ischemia [1]. Post-treatment CT evaluates HCC response to therapy and detects new HCCs.

In this chapter, we will review the CT protocol for the cirrhotic liver, the CT features of HCC before and after treatment, and the CT features of portal vein thrombosis.

26.2 CT Protocol

The use of state-of-the-art equipment and of a tailored protocol is crucial for optimal HCC detection and staging. Eight-detector row CT scanner and 5-mm slice thickness are considered the minimal technical requirement to obtain high-quality images [1, 2]. Intravenous, bolus injection (4–5 cc/s) of an iodinated contrast material is mandatory to characterize focal liver lesions and to evaluate hepatic vessels. The minimum iodine concentration of intravenous contrast material should be 300 mg per ml [2]. The injection of a saline solution is strongly recommended because it reduces the dose of contrast material remaining in the dead space, and the arrival time of contrast material in the hepatic arteries [3]. The bolus-tracking technique and use of an automated power injector are recommended to obtain a properly timed hepatic arterial phase [2].

The complete CT protocol for a cirrhotic liver includes unenhanced phase, late hepatic arterial phase, portal venous phase, and 3-min delayed phase (Fig. 26.1). Nevertheless, the acquisition of unenhanced images is sometimes considered optional [2], and most centers do not routinely acquire unenhanced phase to reduce patient exposure to ionizing radiations. This is because unenhanced images do not significantly improve HCC detection [4]. Unenhanced phase is helpful to evaluate Lipiodol distribution in HCC treated with TACE. Moreover, it ensures that calcifications and siderotic regenerative nodules (RNs) that appear of greater attenuation than the liver are not mistaken for enhancing nodules on hepatic arterial phase.

Late hepatic arterial phase is acquired 35 s after contrast injection or, when bolus-tracking technique is used, 18 s

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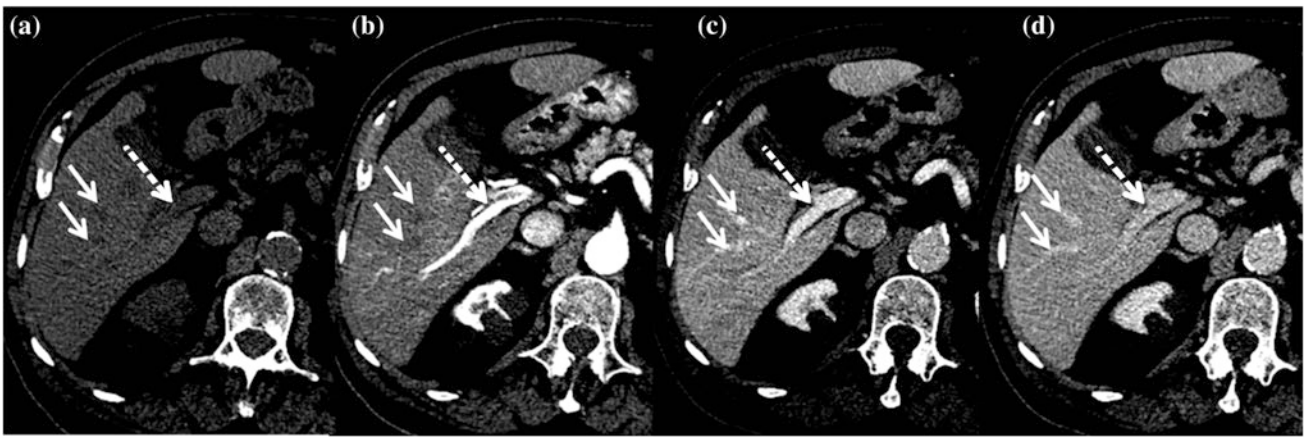

Fig. 26.1 Contrast-enhanced CT protocol of the liver. **a** Unenhanced phase shows hypoattenuation of intrahepatic portal vein branches (*dotted arrow*) and hepatic veins (*arrows*). **b**. Hepatic arterial phase image shows moderate enhancement of intrahepatic portal vein branches and absent enhancement of hepatic veins. **c**. Portal venous phase image shows simultaneous enhancement of portal and hepatic veins, and maximal enhancement of the liver parenchyma. **d**. Delayed phase image shows a decreased attenuation of the portal and hepatic veins, and the liver parenchyma

after the trigger threshold (120–150 HU) is reached at the level of the suprarenal abdominal aorta. Moderate enhancement of the intrahepatic portal vein branches and absent enhancement of hepatic veins suggests an appropriate timing [5]. Hepatic arterial phase is crucial to evaluate HCC enhancement and hepatic arterial anatomy. The portal venous phase is acquired approximately 60–70 s after contrast injection. At this time, the portal and hepatic veins show simultaneous enhancement, and the liver parenchyma shows maximal enhancement [5]. Portal venous phase is crucial to detect HCC venous wash-out and evaluate patency of portal venous system and hepatic veins. On 3-min delayed phase, the liver parenchyma attenuation decreases, while the portal and hepatic veins remain enhanced but to a lesser degree than in portal venous phase. These findings reflect contrast diffusion into extracellular compartments, and start of urinary excretion [6]. Delayed phase acquisition is useful to detect wash-out in some of those cases where HCC is still isoattenuating on portal venous phase.

Multiplanar reformation (MPR) and three-dimensional reconstruction with maximum intensity projection (MIP) can help radiologists to evaluate hepatic vascular anatomy and HCC relationship with surrounding structures.

26.3 Cirrhotic Nodules

HCC is usually the result of multistep carcinogenesis, from RN, to dysplastic lesions (dysplastic foci and dysplastic nodule (DN)—low and high grade), followed by early and progressed HCC [7, 8]. During this process, blood supply changes: the intranodular portal flow gradually decreases, while the arterial flow gradually increases [9, 10]. Hence, CT differentiation of HCC from non-malignant RN and DN is based mainly on the evaluation of tumor vascularity [9, 10]. Less commonly, HCC can develop without intermediate histologically identifiable steps ("de novo hepatocarcinogenis") [11].

26.3.1 Regenerative Nodules

Regenerative nodules are a localized proliferation of normal hepatocytes surrounded by fibrous septa [12]. Typically, RNs are innumerable, and measure less than 5 mm in diameter [12]. RNs contain one or more portal tracts. Newly formed (unpaired) arteries are not present [12]. Although RNs are present in all cirrhotic livers, they are rarely diagnosed at CT [13]. At unenhanced CT, RNs usually show an attenuation similar to that of the surrounding liver [13]. Uncommonly, RNs can show hyperattenuation due to iron content (siderotic nodules). RNs do not enhance on hepatic arterial phase, and are typically occult on contrast-enhanced CT images [14]. The detectability of RNs on contrast-enhanced CT images is predominantly related to the size of the nodules and the degree of enhancement of surrounding fibrosis, the so called "lace-like hepatic fibrosis", thus RNs are clearly identifiable only in advanced and macronodular cirrhosis [14].

26.3.2 Dysplastic Nodules

Dysplastic nodules are distinct or vague parenchymal nodules composed of hepatocytes with evidence of dysplasia [15]. DNs are typically larger than RNs, and can be single or

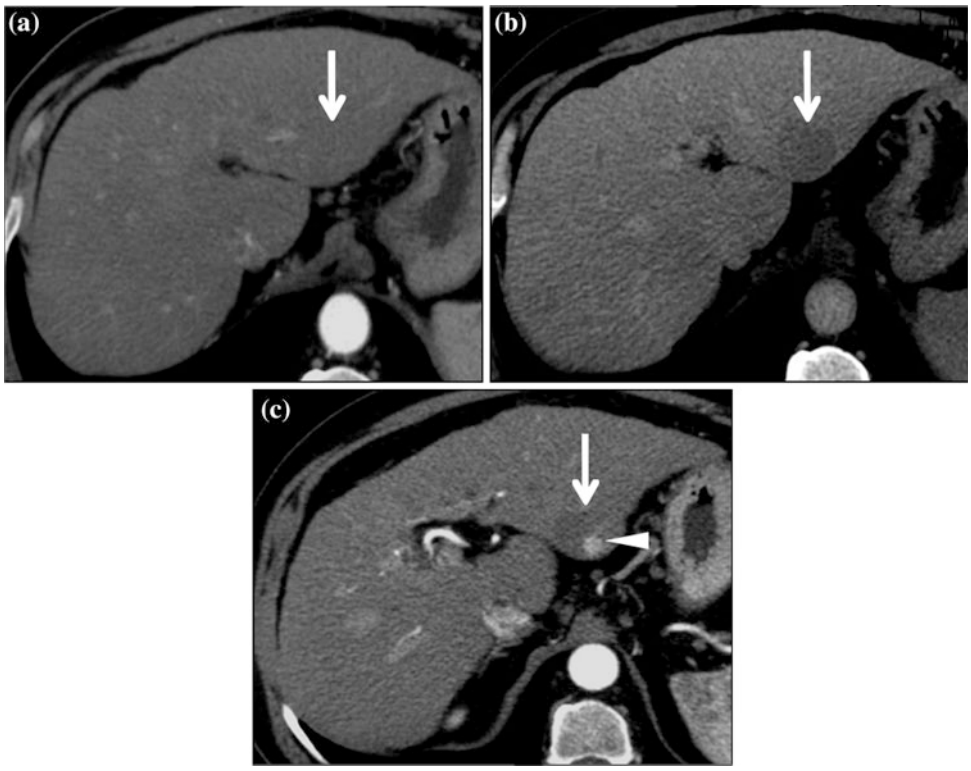

Fig. 26.2 Hepatocellular carcinoma with nodule-in-nodule appearance in a 61 year-old man with HBV-related hepatic cirrhosis. **a–b** On contrast-enhanced CT scan the dysplastic nodule (*arrow*) shows iso to hypoattenuation on hepatic arterial phase (**a**) and hypoattenuation on portal venous phase (**b**). Hepatic arterial phase CT scan eight months after a-b shows an enhancing nodule (*arrowhead*) within a bigger hypoenhancing nodule (*arrow*), consistent with nodule-in-nodule appearance

multiple [15, 16]. Histologically, there are two types of DNs: low grade DNs and high grade DNs [15]. The former do not show cytological and architectural atypia, and are morphologically similar to RNs [15]. The latter show cytological and architectural atypia but no definite malignant changes [15]. Detection of high grade DNs is important for the management of cirrhotic patient because of the risk of malignant transformation. DNs are usually isoattenuating to the liver on unenhanced CT.

Siderotic DNs can show hyperattenuation on unenhanced CT. DNs are typically iso or hypoenhancing to the liver [17]. Less commonly, DNs show arterial enhancement [17]. The enhancement pattern reflects intranodular blood supply: DNs represent an intermediate step in multistep hepatocarcinogenis, and are supplied by both residual portal tracts (containing the portal vein and normal hepatic artery) and unpaired arteries [15, 17].

26.3.3 Nodule-in-Nodule

The "nodule-in-nodule" appearance represents a small HCC within a larger DN. During the multistep hepatocarcinogenesis process, one or more tiny foci of HCC can develop within a high grade DN. The foci of HCC grow rapidly, and replace completely the surrounding DN [18]. CT can occasionally detect these foci of HCC. The "nodule-in-nodule" appearance represents a small hypervascular subnodule (representing the HCC) within a hypovascular nodule (representing the DN) (Fig. 26.2). These findings support the multistep hepatocarcinogenesis theory in vivo.

26.3.4 Hepatocellular Carcinomas

HCCs are malignant tumors composed of cells with hepatocellular differentiation [12]. HCCs are pathologically classified as early and progressed. Early HCCs represent the incipient stage of hepatocarcinogenesis [15]. Early HCCs measure less than 2 cm in diameter, and are composed of well-differentiated neoplastic cells [15]. Early HCCs contain few unpaired arteries, and retain some portal tracts [15]. Progressed HCCs represent the final stage of hepatocarcinogenesis. Progressed HCCs have a distinct nodular appearance, are usually moderately differentiated, and may invade the vessels; the latter finding differentiates them from early HCCs. Blood supply is almost exclusively from unpaired arteries, and the size is variable. Progressed HCCs

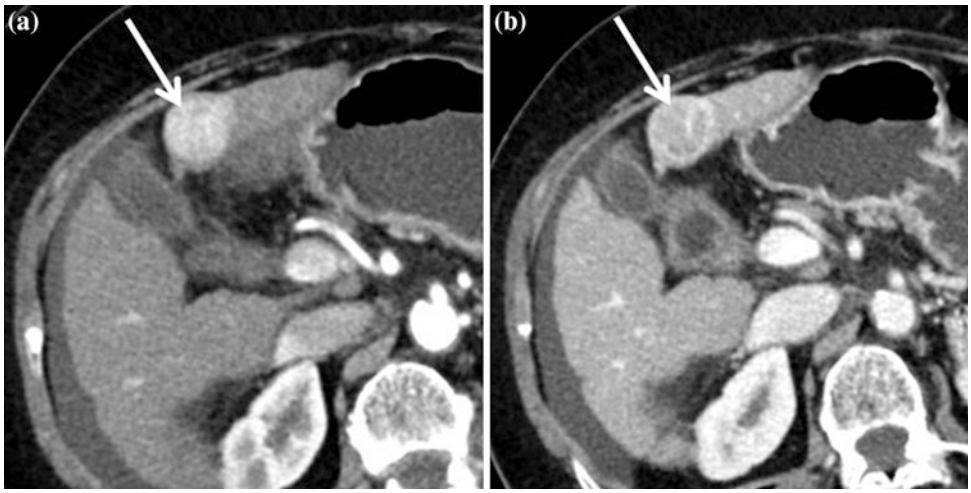

Fig. 26.3 HCC with typical CT features in a 77-year-old man with HCV-related cirrhosis. CT scan shows an HCC (*arrow*) with enhancement on hepatic arterial phase (**a**) and wash-out *(arrow)* on portal venous phase (**b**). HCC is surrounded by a capsule that enhances on portal venous phase

are classified into small (≤ 2 cm in diameter) and large (>2 cm in diameter). Imaging appearance depends on HCC size and grade of differentiation. HCCs are usually iso or hypoattenuating to the liver on unenhanced phase. After intravenous contrast administration, HCCs typically show moderate arterial enhancement and venous wash-out [1] (Fig. 26.3). Arterial enhancement (hypervascularity) is defined as hyperattenuation of a lesion compared with the surrounding liver on arterial phase [5]. The basis of arterial enhancement is well understood: HCCs are predominantly perfused from newly formed arteries, while the surrounding liver is perfused by both the hepatic artery (25 % of blood supply) and the portal vein (75 % of blood supply) [17, 19]. Arterial enhancement is homogeneous in small HCCs and heterogeneous in large HCCs. Venous wash-out is defined as hypoattenuation of a lesion on portal venous and/or delayed phase compared with the surrounding liver [5]. The mechanism underlying venous wash-out is multifactorial, and results from a combination of early venous drainage through perinodular hepatic sinusoids and portal veins, HCC decreased portal supply, and progressive enhancement of cirrhotic liver [20]. Venous wash-out is more frequently appreciated on delayed phase than on portal venous phase [21]. The combination of arterial enhancement and venous wash-out has high diagnostic accuracy for the diagnosis of HCC in at high risk patients [22]. The sensitivity and specificity are approximately 100 % for large HCCs, and are lower in smaller HCCs (the bigger the HCC, the higher the diagnostic accuracy). Thus, most current guidelines recommend that a noninvasive, imaging-based diagnosis of HCC can be made in at risk patients if a lesion shows arterial enhancement and venous wash-out. Western HCC guidelines require a minimum lesion diameter of one cm [1, 23], while asian guidelines do not take into account lesion size [23]. Because most guidelines are based only on evaluation of tumor vascularity, most early HCCs and small progressed HCCs are underdiagnosed [24]. The former posses a low number of unpaired arteries, and are essentially hypovascular lesions. The latter have an increased number of unpaired arteries, but retain portal tracts, and, therefore, can enhance on hepatic arterial phase, but lack venous wash-out [24].

A peritumoral capsule is typically observed in progressed HCCs [25], and is considered a characteristic feature of HCC [5]. Peritumoral capsule is composed of an inner thin layer containing fibrous tissue, and an outer thick layer containing small vessels and biliary ducts [26]. Peritumoral capsule is considered as a positive prognostic factor of HCC: encapsulated HCCs exhibit a lower recurrence rate after hepatic resection and a better response to trans-arterial chemioembolization (TACE), compared to nonencapsulated HCCs of similar size and grade [27, 28]. At CT, the capsule shows iso to slight hypoattenuation on unenhanced phase, and progressive enhancement from arterial to delayed phase [26]. Ancillary features for the diagnosis of HCC include intralesional fat, mosaic appearance, nodule-in-nodule appearance, and corona enhancement [5]. Ancillary features do not allow a definitive diagnosis of HCC, but can help radiologists to characterize indeterminate cirrhotic nodules. Intralesional fat is a common finding of early HCC, and becomes infrequent with increasing tumor diameter and histologic grade [15, 29]. However, as intralesional fat can be also detected in high grade DNs and, occasionally, in low grade DNs, it should be used with caution to confirm the diagnosis of HCC [15, 30]. The term corona enhancement

indicates a ring-like arterially enhancing area at the periphery of an arterially enhancing HCC, which fades on portal venous and delayed phases [31, 32]. This area corresponds to HCC draining area [31, 32]. Mosaic appearance is characteristically observed in large HCCs and is due to internal areas with different composition (hemorrhage, calcifications and necrosis), and enhancing septa [2]. Infiltrative or diffuse type HCC accounts for up to 20 % of HCCs and consists of innumerable, minute HCC nodules, which spread into multiple hepatic segments with an infiltrative growth pattern [33]. Infiltrative HCC shows minimal or no enhancement on hepatic arterial phase, and a slight hypoattenuation on portal venous and delayed phases [34]. Portal vein invasion is a common finding [34].

26.4 Portal Vein Thrombosis

Portal vein thrombosis is defined as a partial or complete filling defect within the vascular lumen [35]. Portal vein thrombosis occurs as a complication of portal hypertension (bland thrombus) or HCC (neoplastic thrombus). Portal vein neoplastic thrombosis indicates HCC macrovascular invasion, and render a patient ineligible to liver transplantation [2]. Neoplastic thrombi show similar enhancement to HCC, and, occasionally, direct HCC extension into the portal vein can be observed [35] (Fig. 26.4). Additional findings that increase the confidence for diagnosis of neoplastic thrombosis are enlargement of vascular lumen, and presence of multiple striated arterial vessels within the thrombus ("thread and streaks sign") [35, 36]. Bland thrombi are usually hypoattenuating on portal venous phase, and do not enhance on hepatic arterial phase [35] (Fig. 26.5). In more severe cases, portal vein narrowing, calcifications and cavernous transformation are observed [35, 36]. Benign portal vein thrombosis is not an absolute contraindication to liver transplantation, but it renders the procedure more difficult, and increases the risk of graft loss and perioperative mortality [37, 38].

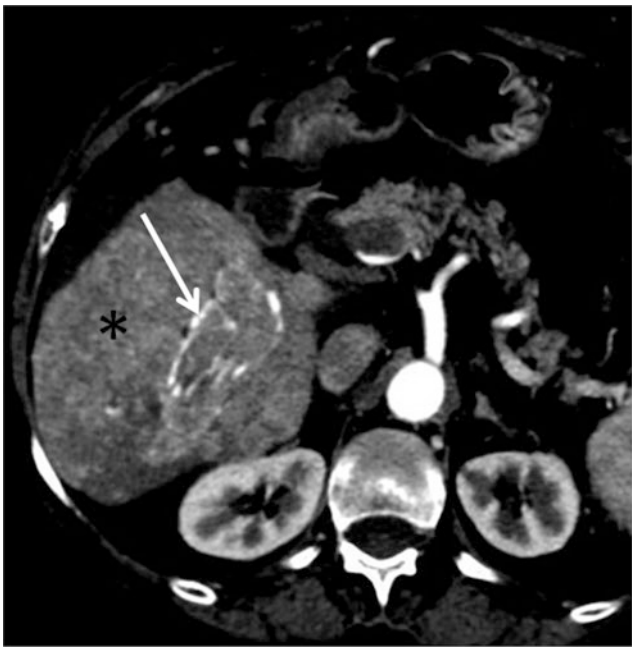

Fig. 26.4 Malignant portal vein thrombosis in a 71-year-old woman with HCV-related cirrhosis and HCC. Hepatic arterial phase CT scan shows a heterogeneously enhancing thrombus *(arrow)* within the right portal vein, and a large HCC in right hepatic lobe (*asterisk*)

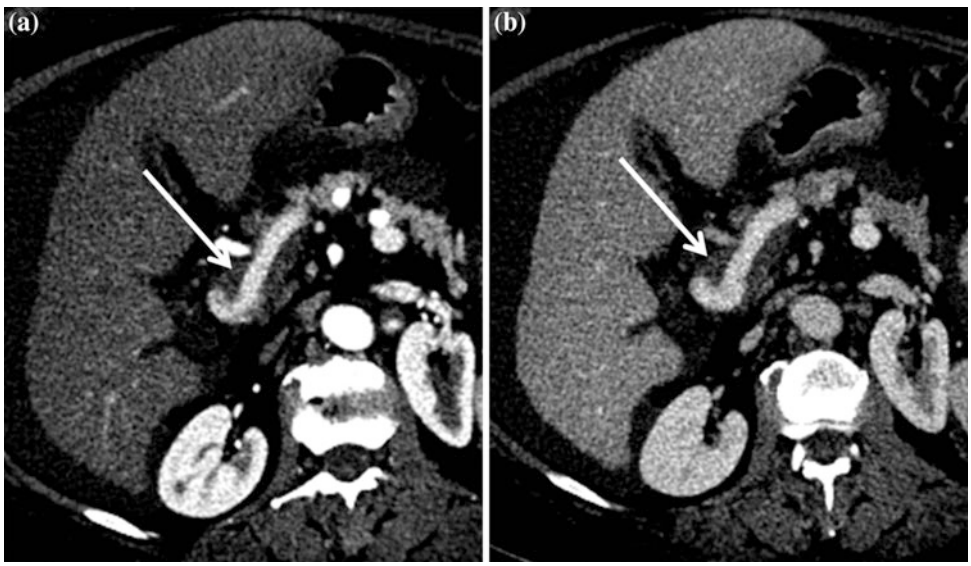

Fig. 26.5 Benign portal vein thrombosis in a 58-year-old man with HCV-related cirrhosis. Hepatic arterial (**a**) and portal venous (**b**) phase CT scan shows a non occlusive, hypoattenuating, non enhancing thrombus within *(arrow)* the main portal vein

26.5 Treated HCC

The scope of HCC treatment is to improve patients' survival and preserve health-related quality of life. Hepatic resection is the preferred treatment modality for HCC, and can be offered to patients with well-preserved liver function (Child–Pugh class A and B) [1]. When hepatic resection is unfeasible (e.g., multiple HCCs with bilobar distribution) or unsafe (e.g., severely impaired hepatic function), image-guided procedures, including radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), and trans-arterial therapies can be considered as a first-line treatment [1]. Targeted therapies are indicated in patients with advanced HCC stage or with progression after treatment, in which hepatic resection and image-guided procedures are not possible [1]. Residual and recurrent disease after HCC treatment, however, is not rare. Thus, CT surveillance plays a crucial role in treatment monitoring. Post-treatment is usually performed at one, 3 and 6 months after treatment, and every 6 months thereafter [38]. One-month follow-up is crucial to detect residual disease and potential post-procedural complications [38]. Later follow-up studies are crucial to detect tumor recurrence, defined as the occurrence of viable tumor in a HCC that has been previously considered completely necrotic at imaging [38]. Evaluation of treatment response is primarily based on the detection of viable tumor rather than changes in tumor size [39]. As a general rule, arterially enhancing areas are presumed to be viable tumor, while absence of arterial enhancement typically means necrotic tumor [38]. CT findings of treated HCC depend on treatment modality [38].

26.5.1 Radiofrequency Ablation

RFA induces HCC coagulative necrosis by placing one or more electrodes within the tumor [40]. RF-ablated area should be larger than the preexisting tumor [40]. RF-ablated area remains stable in size or shrinks with time [41]. Absence of arterial enhancement within the treated area indicates a successful RFA [42]. A circumferential, thin, arterially enhancing rim, however, can be sometimes observed along the margins of the treated area [42]. This rim is due to RFA-induced inflammatory reaction and usually disappears with time [43]. Viable tumor must be suspected if arterially enhancing area is nodular or irregular and the treated area does not encompass the preexisting tumor [42] (Fig. 26.6). Treatment-related complications include abscess within the treated area, wedge-shaped arterially enhancing area in proximity of the treated area due to iatrogenic arteriovenous shunts, portal vein thrombosis, and tumor seeding [42].

26.5.2 Percutaneous Ethanol Injection

PEI induces tumor coagulative necrosis by percutaneous injection of ethanol in the HCC [44]. A successful treated HCC does not enhance on hepatic arterial phase, and shows hypoattenuation on contrast-enhanced images [44]. Similarly to RF-ablated HCC, successfully treated area remains stable in size or shrinks with time. PEI results in a lower treatment response, as compared with RFA [45, 46]. Treatment-related complications (e.g., abscess within the treated area, bile ducts dilatation due to biliary injury, peritoneal bleeding and tumor seeding) are extremely rare [44].

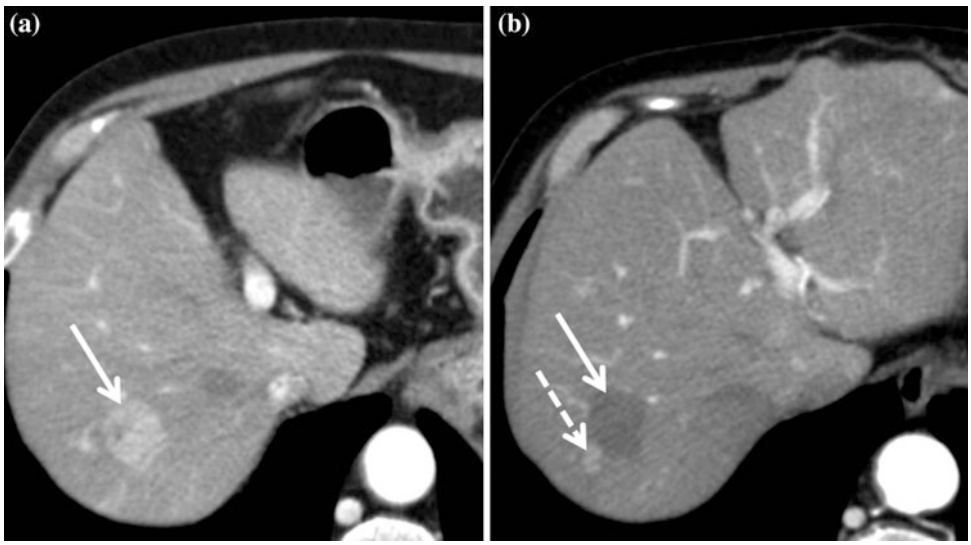

Fig. 26.6 Recurrent HCC after RFA in a 66-year-old man with HCV-related hepatic cirrhosis. **a** Pre-RFA hepatic arterial phase CT scan shows an enhancing HCC (*arrow*). **b** Hepatic arterial phase CT scan obtained five months after RFA shows an enhancing nodule (*dotted arrow*) along the margins of the RF-ablated area (*arrow*) representing recurrent disease

26.5.3 Trans-arterial Therapies

Traditional trans-arterial chemoembolization (TACE) consists of trans-arterial infusion of chemotherapeutic agents mixed with Lipiodol (Andre Guerbet, Aulnay-sous-Bois, France) into HCC feeding vessels [47]. The treated area has the same size of the preexisting HCC, and remains stable in size or shrinks with time. CT assessment of TACE efficacy relies on the evaluation of Lipiodol uptake and vascularization [48]. Specifically, any HCC portion, which retains Lipiodol is considered to be necrotic tissue, while any area within the HCC or along its margins, which shows arterial enhancement and venous wash-out, is considered to be viable tissue [48] (Fig. 26.7). As Lipiodol shows spontaneous hyperattenuation, its uptake is primarily evaluated on unenhanced phase. Lipiodol uptake, however, can cause beam-hardening artifacts that make difficult the detection of enhancing viable tumor [49]. Treatment-related complications include hepatic abscess, wedge-shaped arterially enhancing area, liver infarction, and iatrogenic dissection of the celiac trunk [50].

TACE with drug-eluting microspheres and ^{90}Y radioembolization represent two emerging alternative techniques to traditional TACE [47]. Drug-eluting microspheres embolize HCC feeding arteries, and release the chemotherapeutic agents into the HCC in a sustained and controlled manner, thus reducing the systemic effects related to the administration of chemotherapeutic agents [47]. The absence of Lipiodol-related beam-hardening artifacts helps detect viable arterially enhancing HCC and evaluate treatment response, compared with traditional TACE [49]. Radioembolization consists of injection of embolic particles loaded with a radioisotope into HCC feeding arteries. Similarly to HCC treated with drug-eluting microspheres, Lipiodol-related beam-hardening artifacts are absent [51]. A peritumoral edema and a thin, arterially enhancing rim can be sometimes observed along the margins of the treated HCC immediately after treatment and disappear with time

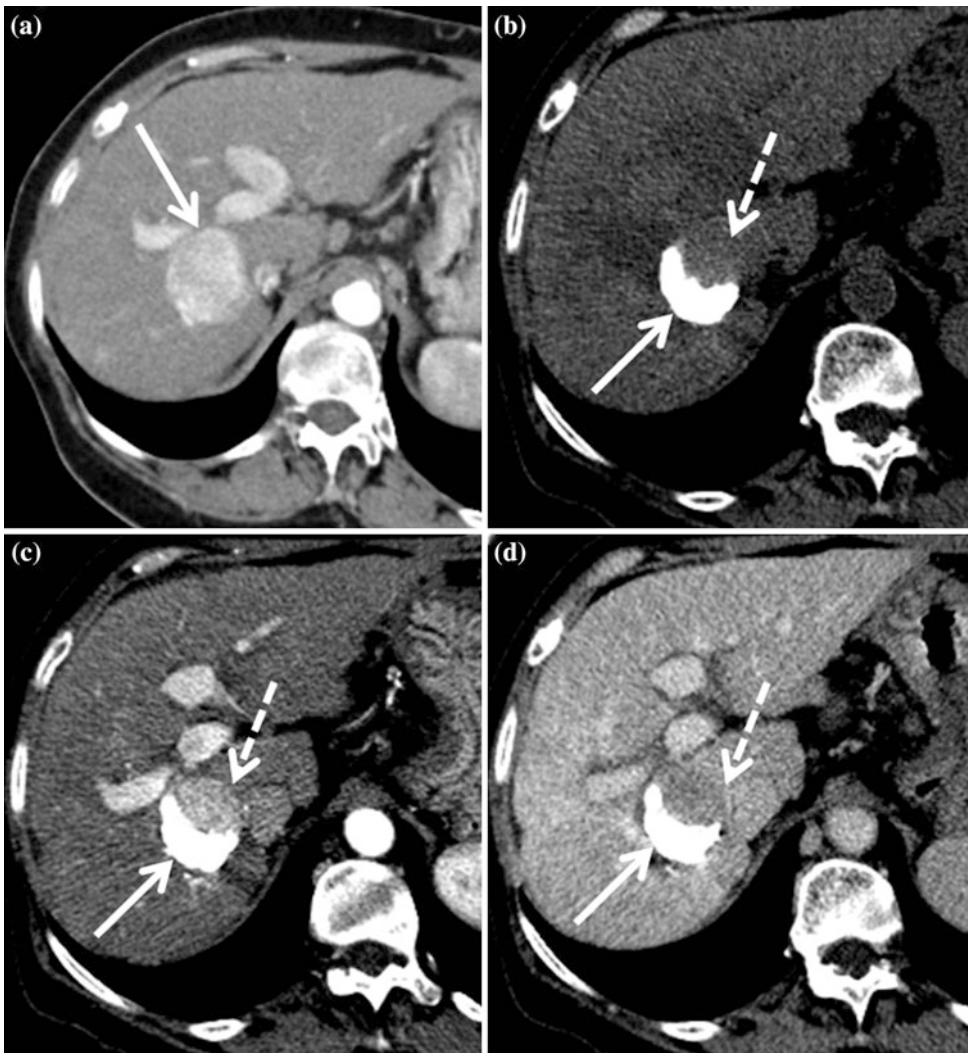

Fig. 26.7 Residual HCC after TACE in a 74-year-old woman with HCV-related hepatic cirrhosis. **a** Pre-TACE hepatic arterial phase CT scan shows an enhancing HCC (*arrow*). **b–d**. On CT scan obtained one month after TACE the residual viable tumor *(dotted arrow)* shows defective Lipiodol retention on unenhanced phase (**b**), enhancement on hepatic arterial phase (**c**) and wash-out on delayed phase (**d**), while the necrotic tumor *(arrow)* shows homogeneous Lipiodol retention on unenhanced phase, and no enhancement on hepatic arterial phase

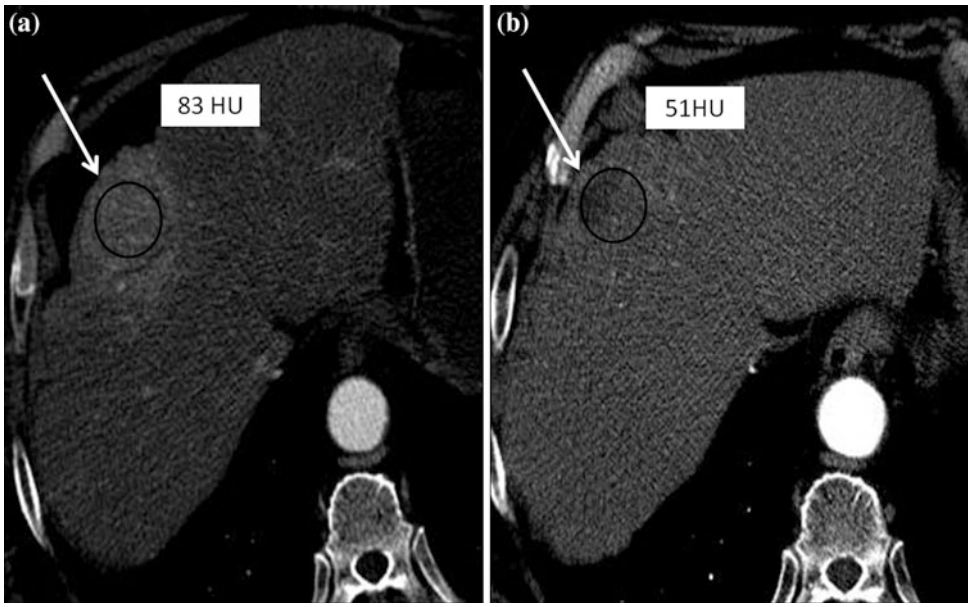

Fig. 26.8 HCC treated with Sorafenib in a 79-year-old woman with HCV-related hepatic cirrhosis. **a** Pre-Sorafenib hepatic arterial phase CT scan shows an enhancing HCC (*arrow*). **b** Hepatic arterial phase CT scan obtained three months after start of Sorafenib administration shows disappearance of HCC enhancement *(arrow)* and decrease of HCC attenuation

[51]. Radioembolization can cause atrophy of the treated lobe [51].

26.5.4 Targeted Therapies

Targeted therapies such as Sorafenib inhibit HCC neoangiogenesis and growth, but do not necessarily cause HCC necrosis [52]. The primary effect of targeted therapies is a decrease in HCC vascularity [52] (Fig. 26.8). Thus, the traditional WHO and RECIST criteria based on evaluation of tumor size can underestimate the real response rate [53]. Assessment of treatment response relies on evaluation of the viable HCC burden, defined as the portion of the HCC that shows arterial enhancement and venous wash-out [39]. Response evaluation criteria include the modified RECIST (mRECIST) criteria and modified Choi criteria. The former evaluate the sum of the diameters of the viable portions of target lesions, while the latter evaluate the changes in hepatic arterial phase attenuation of the target lesions before and after therapy [39, 54].

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Clinical Features and Clinician's Diagnostic Approach to Hepatocellular Carcinoma

27

Tamar Thurm and Oren Shibolet

Contents

27.1 Introduction

Hepatocellular carcinoma (HCC) is the most common form of liver cancer. It is the fifth most common cancer worldwide and the third most common cause of cancer-related death globally. Most cases of HCC (approximately 80 %) are associated with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections [1]. Although HCC has historically been more common in the developing world, its incidence in developed countries has almost tripled since the early 1980s, largely as a result of the increased incidence of liver cirrhosis [2, 3]. This incidence is increasing because of the long-term consequences of HCV infection, the obesity epidemic and non-alcoholic steatohepatitis (NASH)-associated cirrhosis, as well as better diagnostic modalities. An estimated 1 million new cases of HCC are diagnosed annually.

HCC is rarely seen during the first 4 decades of life, except in populations where HBV infection is hyper-endemic such as in South East Asia and Sub-Saharan Africa [4]. The mean age at diagnosis with HCC is 63–65 years in Europe and North America. HCC is predominant among men, with the highest male: female ratios in areas of South East Asia [1].

Eighty to ninety percent of patients with HCC have underlying chronic liver disease and cirrhosis. Approximately 1–8 % of these patients will develop HCC per year depending on etiology, with the highest risk seen in patients with HCV-associated cirrhosis (3–8 % yearly) [5]. Advanced liver disease, as manifested by platelet count of less than 100,000, presence of esophageal varices, ascites and encephalopathy, in addition to older age and male gender, correlates with a higher risk to develop HCC in cirrhotic patients [6]. Studies have shown that liver cancer incidence increases in parallel to portal hypertension as measured by hepatic venous pressure gradient (HVPG) [7] or to the degree of liver stiffness as measured by transient elastography [8, 9].

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Table 27.1 Symptoms and signs of hepatocellular carcinoma

Symptoms	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 Fever	25–41 11–54

Reprinted from Kew [12], p. 1578

The clinical presentation of HCC is variable and can range from an asymptomatic presentation to tumor rupture with a catastrophic hemoperitoneum [10]. Hence, screening and surveillance is of utmost importance in "at-risk" populations to diagnose early HCC and offer potentially curative treatments.

Diagnosis and management of HCC in cirrhotic patients is complex, and requires astute clinical decision-making. It relies primarily on imaging modalities, serum markers, and histology. Patients with HCC are unique, because they combine the complexity of the underlying cirrhosis and that of the malignancy. Clinicians play a pivotal role in performing diagnostic testing and in both the primary and the secondary chemoprevention of HCC and require advanced knowledge of both hepatology and oncology.

This chapter will discuss 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 doctors taking care of patients with HCC.

27.2 Clinical Features

27.2.1 Asymptomatic HCC

Most cases of HCC appear in the setting of cirrhosis; hence, presenting symptoms will be similar to those observed in patients with advanced liver disease. Partially due to screening programs for cirrhotic patients and also to the wide spread use of ultrasonography (US), some tumors are now being detected at an asymptomatic stage. These tumors tend to be small and therefore are more amenable to potentially curative therapies such as resection, transplantation, and tumor ablation [11].

27.2.2 Symptomatic HCC

The classical triad for presentation of HCC, though uncommon in clinical practice, includes right upper quadrant abdominal pain, weight loss, and hepatomegaly (see Table 27.1). The pain is frequently described as a dull, continuous ache that intensifies late in the course of the illness and my radiate to the shoulder. This occurs due to involvement of Glisson's capsule. Firm, often massive hepatomegaly is also a feature of symptomatic malignant liver tumors. Systemic symptoms such as weight loss, fatigue, and anorexia are common in patients with advanced disease.

27.2.3 Hepatic Decompensation Due to HCC

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, progressive encephalopathy, or jaundice. Although hepatic decompensation may be due to "natural" progression of the underlying liver disease, any of the above features should raise the suspicion for new HCC in the differential diagnosis.

27.2.4 Portal Vein Thrombosis

Portal vein thrombosis (PVT) is a common complication of HCC, complicating 34–50 % of cases and has a markedly deleterious effect on prognosis. To distinguish PVT occurring due to malignant spread into the portal vein, it is also called portal vein tumor thrombus (PVTT) [13]. Untreated patients with PVTT have a life expectancy of less then 3 months. In a treated patient the prognosis is heterogeneous, depending on patient and tumor characteristics [13].

Diagnosis is based on imaging that may show vascular enhancement of the thrombus with occasional traversing blood vessels. Differentiating PVT from PVTT is crucial because liver transplantation is contraindicated with macrovascular invasion. A few reports suggest using Endoscopic US (EUS) guided Fine Needle Aspiration (FNA) from PVTT for HCC pathologic diagnosis [14].

27.2.5 Gastrointestinal Hemorrhage

Approximately 10 % of patients with HCC will present with some form of gastrointestinal bleeding as their first manifestation of the tumor. About 45 % of these patients will have esophageal variceal hemorrhage. Although not necessarily associated with HCC, this may occur due to PVTT from direct tumor invasion causing elevated portal pressure.

Peptic ulcer disease, portal hypertensive gastropathy, and other causes for gastrointestinal bleeding account for the remaining 55 % of cases [15]. Rarely, the tumor may invade directly into the gastrointestinal tract and cause significant bleeding at presentation [16].

27.2.6 Tumor Rupture/Hemoperitoneum

HCC can manifest as an "acute abdomen" when the tumor ruptures, causing a hemoperitoneum. Tumor rupture may occur spontaneously or with minor blunt abdominal trauma. Spontaneous tumor rupture is one of the most severe complications of HCC. The clinical presentation is that of severe abdominal pain, vascular collapse, and signs of peritoneal irritation. Rupture is mainly a consequence of increased tension from tumor progression, central necrosis, or liquefaction [17]. It was suggested that increased intratumoral pressure occurs due to progressive or sudden occlusion of the hepatic veins by tumor invasion causing venous congestion. Computed Tomography (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, or the "enucleation sign" with findings of a low attenuated mass with peripheral rim enhancement [18].

The following findings are associated with an increased risk of rupture: a large HCC, a contour protrusion, and PVT [18].

Hemostasis is the primary concern and resection of the tumor is secondary [19]. In hemodynamically stable patients, one-stage hepatectomy has shown better results. However, in hemodynamically unstable patients, transarterial embolization (TAE) and surgical hemostasis are the first choice of action followed by two-stage tumor resection in a stabilized patient, pending a careful evaluation of functional liver reserve, coagulopathy, tumor size, and location [19].

Sometimes the presentation of tumor rupture is more insidious and is suspected upon discovery of hemorrhagic ascites. Hemorrhagic ascites can occur spontaneously and from other causes, but is most commonly caused by HCC. Patients with hemorrhagic ascites have higher rates of spontaneous bacterial peritonitis, acute kidney injury, and are more likely to require hospitalization in an intensive care unit. They have higher mortality rates than patients with non-hemorrhagic ascites [20].

27.2.7 Paraneoplastic Syndromes

These systemic presentations result, directly or indirectly, from synthesis and secretion of biologically active substances such as hormones or hormone-like substances by the tumor. Physical findings associated with hormonal oversecretion in a cirrhotic patients, should raise a clinical suspicion of a paraneoplastic syndrome and merit a search for HCC (see Table 27.2).

Paraneoplastic syndromes associated with HCC are exceedingly rare, with limited literature coming from mostly isolated case reports and will be discussed briefly.

Two types of *Hypoglycemia* (<5 % of patients) can be seen in HCC patients. Type A hypoglycemia occurs with rapidly growing tumors in markedly emaciated patients with significant muscle wasting. The mechanism is attributed to the inability of the liver, largely replaced by a tumor, to satisfy glucose demands of the tumor and other tissues.

Table 27.2 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 [12], p. 1579

These patients have suppressed insulin and c-peptide levels and increased glucagon, which is due to hypoglycemia-induced counter-regulatory mechanisms. Type B hypoglycemia, which represents only 5–13 % of paraneoplastic hypoglycemia in HCC, manifests as severe hypoglycemia early in the course of the disease. It results from defective processing of the precursor to IGF2 (pro IGF2) by the hepatocytes. IGF2 circulates as smaller particles, which transfer more easily through capillary membranes and have more access to IGF1, IGF2, and insulin receptors, thereby causing increased glucose uptake [21]. *Polycythemia* (<10 % of patients) is caused by synthesis of an erythropoietin-like substance by malignant hepatocytes [22]. Patients with HCC, especially the sclerosing variety, may present with *hypercalcemia* in the absence of osteolytic metastases. This is caused by production of parathyroid hormone-related peptide (PTHrP) by tumor cells [23]. *Arterial hypertension* complicating HCC is the result of ectopic synthesis of angiotensinogen by malignant hepatocytes [24]. *Feminization*, results from the tumor's conversion of circulating dehydroepiandrosterone to estrone, and, to a lesser extent, estradiol [25]. *Hypercholesterolemia* is the result of autonomous de novo synthesis of cholesterol by the tumor [26]. *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 [27].

27.2.8 Cutaneous Manifestations

Several *cutaneous manifestations* have been described in association with HCC; however, none is pathognomonic. These include dermatomyositis, pemphigus foliaceus, sign of Leser–Trelat, pityriasis rotunda, and porphyria cutanea tarda [28]. 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 [29].

27.2.9 Other Rare Manifestations

HCC can cause fever of unknown origin. This is possibly due to the release of pyrogenic cytokines either directly from tumor cells or from macrophages reacting to the tumor. Suspected culprits include interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)α. Other substances include prostaglandin E2 which acts on the hypothalamus, causing a change in the thermostatic set point [30].

Massive tense ascites resulting from hepatic vein spread (Budd–Chiari syndrome) [31] and obstructive jaundice resulting from bile duct compression are complications of locally advanced tumor.

27.2.10 Metastatic HCC

Metastatic spread is uncommon in HCC. Most cases of metastatic spread occur in patients with advanced intrahepatic HCC. The lung and lymph nodes are the most common sites for metastatic spread accounting for 47 and 45 % accordingly, followed by musculoskeletal disease in 37 % and adrenal involvement in 12 % [32]. The brain is a rare site for metastatic spread, accounting for only 2 % of metastatic disease [33].

27.3 Screening for HCC

In 70–90 % of all cases, HCC develops against a background of chronic liver disease with inflammation and cirrhosis [34]. Major causes of cirrhosis are HBV, HCV, NASH, and alcohol, which account for the vast majority of HCC cases world wide.

Less common causes of HCC include hereditary hemochromatosis (HH)-induced cirrhosis, in which the incidence of HCC is 8–10 % and the tumor accounts for as many as 45 % of deaths [35]. Cirrhosis associated with other chronic liver diseases such as autoimmune hepatitis, alpha-1 antitrypsin deficiency, and primary biliary cholangitis (PBC) has 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. A recent study has shown that Wilson's disease, even in a setting of cirrhosis, is not associated with an increased risk for HCC [36].

In Asia and Africa, exposure to dietary aflatoxin is an important risk factor for HCC, both as an independent risk and as a cofactor in chronic HBV infection [37]. In Sub-Saharan Africa, human immunodeficiency virus infection is recognized as a frequent cofactor that increases the risk of HCC in patients with chronic HBV or HCV infection [38]. The risk of HCC is higher in males, patients older than 50 years and with increased α-fetoprotein (AFP) concentration. Smoking slightly increases the oncogenic risk [39], whereas coffee consumption seems to reduce the risk [40].

HCC surveillance is associated with significant improvement in early tumor detection and receipt of curative therapies [41]. Unfortunately, screening programs have not been successful in increasing overall survival in this deadly cancer. In a recent review, El-Serag et al. assessed the probabilities of various assumptions in a surveillance

algorithm. Independent probabilities in HCC screening include: HCV or HBV diagnosis rates (80 %), access to surveillance (80 %), recommendation for surveillance (80 %), acceptance by patients (80 %), adherence to recommended intervals (80 %), proper follow-up (80 %), and availability of diagnostic and therapeutic options (80 %). If these probabilities are multiplied and considering the estimated efficacy of HCC surveillance in clinical trials is 35 %, the resulting effectiveness could be as low as 6 % [42]. A survey of 436 HBV-infected Korean participants, showed that only 27 % were up to date with HCC screening and more than half (52.9 %) have never been screened [43]. Even more alarming results were published in a study assessing the prevalence of HCC surveillance among HCV-infected patients with cirrhosis in the Veterans Affairs health care facilities in the United States. In this retrospective cohort study of 126,670 patients with HCV, 10 % had cirrhosis, approximately 42.0 % of patients with cirrhosis received one or more HCC surveillance test within the first year after diagnosis; a decline in the rates of surveillance was observed in the following 2–4 years. Routine surveillance occurred in 12.0 %, inconsistent surveillance in 58.5 %, and no surveillance in 29.5 % [44].

Cost-effectiveness studies indicate that US alone or in association with AFP is the most cost-effective surveillance method. Screening should be implemented to detect HCC at an early stage of cirrhosis and it is likely to be cost ineffective as the liver disease progresses limiting the ability to treat, or after liver transplantation, where the risk markedly declines [45]. Surveillance may be associated with a modest gain in quality-adjusted life years at acceptable costs. A recent study demonstrated that optimal adherence to HCC screening would increase life expectancy by 31 months and decrease HCC mortality at 5 years by 20 % in patients with compensated HCV-related cirrhosis [46].

Surveillance is recommended in target populations of cirrhotic patients and non-cirrhotic chronic HBV carriers (see Table 27.3). The efficacy and cost-effectiveness of surveillance in non-cirrhotic HCV carriers are unclear. A recent study from the United States pointed out that HCC can occur in patients with chronic HCV and bridging fibrosis in the absence of cirrhosis (Metavir F3) [6]. Whether it is cost-effective for these subjects to undergo routine surveillance has not been determined, but on the basis of prior cost-effectiveness analyses they would fall below the 1.5 %/year incidence threshold for initiation of surveillance in an "at-risk" population.

The fact that the transition from advanced fibrosis to cirrhosis cannot be accurately defined led the European Association for the Study of the Liver (EASL) to expand its recommendation of surveillance to include HCV patients with F3 fibrosis. Japanese guidelines extend this recommendation to all patients with chronic viral hepatitis and nonviral cirrhotic patients [4, 47, 48].

Table 27.3 Group for whom HCC surveillance in recommended or in whom the risk of HCC is increased, but in whom efficacy of surveillance has not been demonstrated

Surveillance recommended		
Population group	Threshold incidence for efficacy of surveillance (>0.25 LYG) (%/year)	Incidence of HCC
Asian male hepatitis B carriers over age 40	0.2	0.4–0.6 %/year
Asian female hepatitis B carriers over age 50	0.2	0.3–0.6 %/year
Hepatitis B carrier with family history of HCC	0.2	Incidence higher than without family history
African/North American blacks with hepatitis B	0.2	HCC occurs at a younger age
Cirrhotic hepatitis B carriers	0.2–1.5	3–8 %/year
Hepatitis C cirrhosis	1.5	3–5 %/year
Stage 4 primary biliary cirrhosis	1.5	3–5 %/year
Genetic hemochromatosis and cirrhosis	1.5	Unknown, but probably > 1.5 %/year
Alpha 1-antitrypsin deficiency and cirrhosis	1.5	Unknown, but probably > 1.5 %/year
Other cirrhosis	1.5	Unknown
Surveillance benefits uncertain		
Hepatitis B carriers younger than 40 (males) or 50 (females)	0.2	<0.2 %/year
Hepatitis C and stage 3 fibrosis	1.5	<1.5 %/year
Non-cirrhotic NAFLD	1.5	<1.5 %/year

The American Association for the study of Liver Disease (AASLD) practice guidelines for HCC screening in high risk populations were updated in 2011 (see Table 27.3) [47]. According to these guidelines surveillance was deemed cost-effective if the expected HCC risk exceeds 1.5 % per year in patients with HCV and 0.2 % per year in patients with HBV.

Surveillance recommendations are based on a seminal randomized controlled trial conducted in China that included nearly 19,000 individuals with chronic HBV infection.

Patients were randomized to screening or routine follow-up. The study reported that surveillance with US and AFP tests, repeated at 6-month intervals, reduced HCC-related mortality rates by 37 % [49].

Numerous studies, most of them in HCV patients [50, 51], have tried to assess the optimal interval of surveillance, mostly concluding that semiannual and annual screening programs were comparable. One study, in HBV patients, demonstrated improved survival with 6 months surveillance intervals compared to 12 months [52]. Therefore, rather than making separate recommendations for patients with HBV or HCV, the AASLD issued a single recommendation that surveillance be undertaken at 6 monthly intervals.

27.4 Diagnostic Approach

If HCC is suspected either clinically or because of abnormal screening results, further investigation by imaging techniques and/or biopsy is required. There are two categories for diagnostic tests: serologic and radiologic. Serologic tests include AFP and other more novel markers, while radiologic diagnosis relays on contrast-enhanced imaging.

27.4.1 Serologic Markers

The most commonly used serologic test to detect HCC is AFP. Produced by the yolk sac and the liver during fetal development, the AFP levels drop after birth. In adults AFP levels may rise in patients with HCC, germ cell tumors and liver metastasis. Studies that combined data on liver US and measurements of serum AFP levels found that approximately 20 % of HCC cases are detected based on isolated increase in AFP level, with a nondiagnostic US [53].

Despite this finding, recent AASLD guidelines recommend omitting AFP in HCC surveillance since it lacks sensitivity and specificity. Thus, according to the recommendations the diagnosis of HCC should rely on radiological appearances and when necessary on histology [47].

A recent review challenges this recommendation. The authors argue that measurements of AFP levels have several advantages; Assays for serum levels of AFP are inexpensive, simple to perform, well standardized, and widely available. The authors also claim that AFP test performance, including reproducibility is high, and has been evaluated in randomized trials, population-based and cohort studies. In contrast, US is operator-dependent, with high inter-observer variability and also lacks sensitivity [54]. Several international societies have not joined the AASLD and EASL and have left AFP part of the surveillance protocol [55, 56].

Des-carboxyprothrombin (DCP) has been evaluated as another serum biomarker for the detection of HCC. Its role in screening has not been validated, but it was shown as a predictor of prognosis in patients with PVT and candidates for living-related liver transplantation (LRLT) [57–59]. A large follow-up study in HCV patients confirmed the lack of efficacy of AFP and DCP as surveillance tests, even when used in combination [53].

Other markers that have been assessed for screening include: the ratio of glycosylated AFP (L3 fraction) to total AFP, alpha fucosidase, glypican 3, and HSP-70 [47].

Novel technologies such as genome-wide DNA microarray, qRT-PCR and proteomic studies have been used in an attempt to identify markers of early diagnosis of HCC, however, to date no such biomarker is available [4].

27.4.2 Radiologic Diagnoses

Given the fact that serologic biomarkers have not had the expected results, HCC surveillance and diagnosis is based on imaging. The radiological test most widely used for surveillance is US. Surveillance US has a sensitivity of 44 % and specificity of 91 %, for the detection of HCC. A recent prospective study, published after the AASLD published their recommendations, suggests that sensitivity is significantly improved to 90 %, with minimal loss in specificity (83 %) when combined with AFP [60].

A small HCC on US may take several appearances. The smallest lesions may be hyperechoic. Other lesions may be hypoechoic, or show a “target lesion” appearance. None of these appearances is specific [47]. If HCC is suspected, advancing to another, more sensitive diagnostic modality is needed.

Diagnostic imaging tests used to detect HCC are either triple/quadruple-phase helical CT or a triple-phase dynamic contrast-enhanced magnetic resonance imaging (MRI). The most common diagnostic feature of HCC during CT scan or MRI is the presence of an arterial-enhancing lesion followed by delayed hypointensity of the tumor in the portal venous phase, so-called the “washout phase” [61]. Recent technical advances have enabled MRI to evaluate tumor cellularity with diffusion-weighted imaging (DWI), tumor vascularity with dynamic subtraction imaging, and the function of normal hepatocytes using hepatocyte-specific contrast agents [62]. Gadolinium-ethoxybenzyl-diethylenetriamine (Gd-EOB-DTPA)-uptake by HCC decreases in parallel with the degree of HCC differentiation [63]. It has also been reported that HCC tends to show a higher signal and lower mean apparent diffusion coefficient value on DWI as the histopathological grade rises [64].

Per-lesion sensitivities, stratified by size, can reach 100 % for both modalities for nodular HCCs larger than 2 cm, are around 45 % for 1–2-cm HCCs and 29–43 % (MR imaging) and 10–33 % (CT) for HCCs smaller than 1 cm [65, 66]. CT

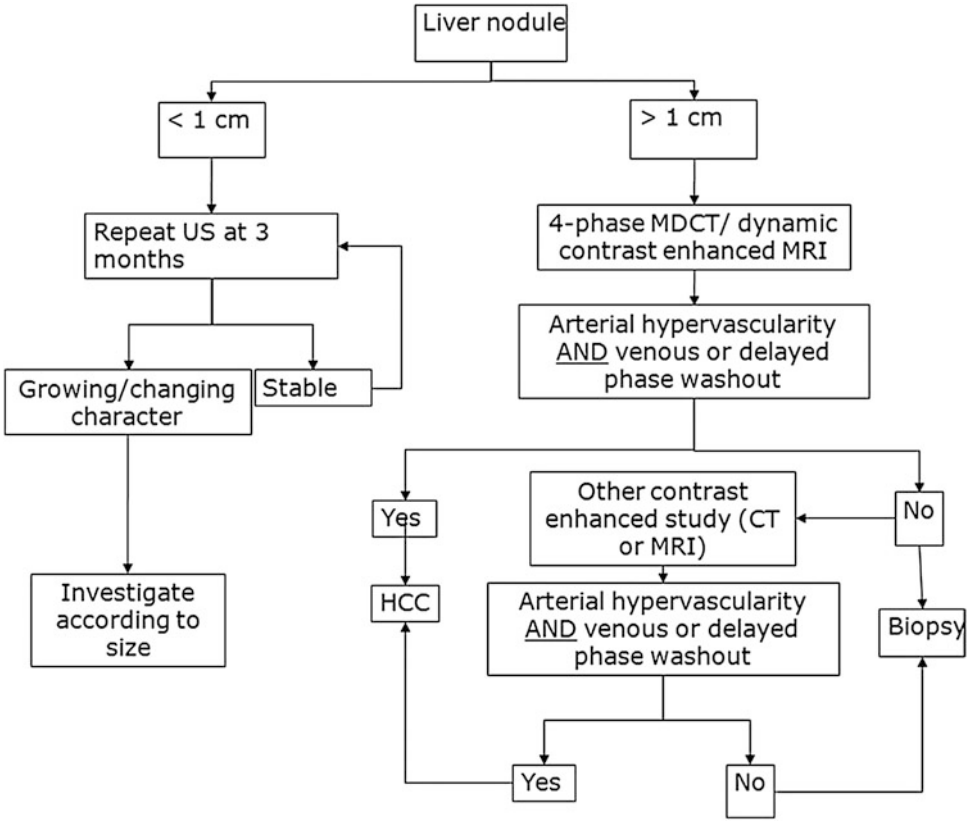

Fig. 27.1 Diagnostic algorithm for suspected HCC. *CT* Computed tomography; *MDCT* Multidetector CT; *MRI* Magnetic resonance imaging; *US* Ultrasound. Reprinted with permission from John Wiley and Sons: Bruix J, Sherman M. Management of hepatocellular carcinoma: An update. Hepatology, 2011

is widely available, rapid, robust, and compared with MR imaging needs less expertise to perform and to interpret. Disadvantages include radiation exposure and relatively low soft-tissue contrast [66].

Contrast-enhanced US (CEUS) is a relatively new imaging modality and has been debated as a diagnostic tool in HCC. The AASLD guidelines did not recommend CEUS as an imaging modality for HCC owing to low specificity. Despite this recommendation it is note worthy that CEUS has been extensively used for more than 10 years for liver imaging in many European and Asian countries with marked success [67].

Current imaging techniques allow detection of liver nodules as small as 0.5 cm on 4 phase multidetector CT or dynamic contrast-enhanced MRI. Noninvasive diagnostic modalities are accurate for the diagnosis of HCC, with a specificity of up to a 100 % in a single modality [68]. Unfortunately, such an absolute specificity has its downside, a low sensitivity [4]. Assessment of tumor extension is critical for defining staging and treatment strategy. Yet underestimation of 25–30 % is expected even with the best state-of-the-art technology [4, 69, 70]. Bone scintigraphy should be used for evaluating bone metastases. PET-based imaging is not accurate to stage early tumors; however, recent data suggests that it may be important in identifying patients with aggressive tumor biology [71].

Masses detected on surveillance require further investigation. Lesions <1 cm should be followed closely with imaging every 3 months in order to detect growth. If there is lack of growth over a period of more than 1–2 years, then routine surveillance should resume at 6-month intervals. Typically appearing lesions above 1 cm in diameter, by either dynamic MRI or multiphase CT require no further investigation for the diagnosis of HCC. If the appearance is atypical for HCC, a second imaging study (complementary CT or MRI) should be performed. If the appearance is typical, the diagnosis of HCC is confirmed. Alternatively, an atypical study merits histological evaluation [47] (see Fig. 27.1).

27.4.3 Staging Systems and Survival Predicting Models

Different staging systems and survival prediction models have been suggested for HCC, mainly to ensure appropriate treatment allocation. The most widely used is the Barcelona Cancer Liver Clinic (BCLC) staging system.

This system (Fig. 27.2) was developed based on the combination of data from several independent studies representing different disease stages and/or treatment modalities. It includes variables related to tumor stage, liver

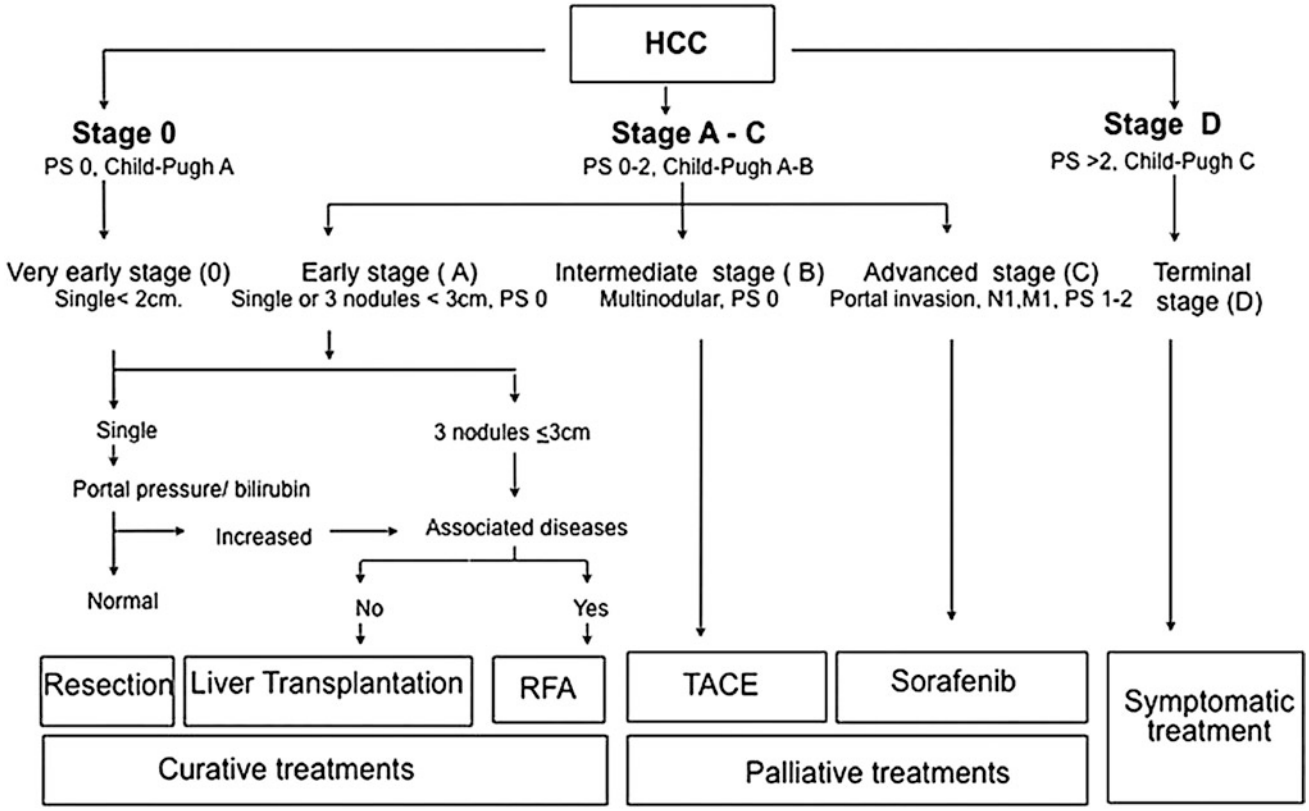

Fig. 27.2 The BCLC staging system and treatment allocation. Reprinted with permission from John Wiley and Sons: Bruix J, Sherman M. Management of hepatocellular carcinoma: An update. Hepatology, 2011

functional status, physical status, and cancer-related symptoms [47, 72]. The main advantage of the BCLC staging system is that it links staging with treatment modalities and with an estimation of life expectancy that is based on published response rates to the various treatments. It identifies those patients with early HCC who may benefit from curative therapies, those at intermediate or advanced disease stage who may benefit from palliative treatments, as well as those at end stage with a very poor life expectancy.

Other staging systems are less frequently used. Some offer only clinical staging with no prognostic value while others are very population specific and not applicable for all HCC patients. These systems and their shortfalls include: The oncologic tumor-node-metastasis (TNM) staging requires pathological information to assess microvascular invasion, available only in patients undergoing surgery. Thus, is nonapplicable in many HCC patients.

Okuda system includes tumor size and severity of cirrhosis (assessment of ascites, serum albumin, and bilirubin levels). It is a clinical score that does not stratify patient by vascular invasion or metastasis.

Cancer of the Liver Italian Program (CLIP) score is a prognostic scoring system for HCC. Tumor-related features are combined (macroscopic morphology, AFP level, and presence of PVT) with an index of cirrhosis severity (Child-Pugh classification) to determine prognosis. It was mostly assessed in Western populations.

The Japanese Integrated Staging Score (JIS score) combines the Child-Pugh grade and the TNM stage. Patients are classified into six groups based on the sum of these scores. Unlike the CLIP score (where no difference between high score group survival is seen), the JIS score offers statistically significant survival difference between almost all JIS scores [73].

Staging systems aim to direct patients to different treatment arms, ensuring optimal utilization of resources. One of the most effective treatments of HCC patients is liver transplantation. In a seminal study Mazzaferro et al. [74] showed that 5-year survival of early stage HCC patients undergoing liver transplantation exceeds 70 %. This established early HCC as a clear indication for liver transplantation in conventional clinical practice. Excellent results could be achieved in patients with solitary HCC < 5 cm or up to 3 nodules smaller than 3 cm (these characteristics are known as the *Milan criteria*). The results from Mazzafero's study and later validations prompted the integration of the Milan criteria into staging systems; transplant indications, and prioritization policies worldwide [75]. There is an ongoing debate in the literature concerning Milan criteria expansion. Studies have shown that a small subset of patients with

tumors outside the Milan criteria have a 50 % 5-year survival rate [76–78], suggesting that the Milan criteria is too strict. Alternative models that have been suggested include the San Francisco [79], Kyoto [80], up-to Seven [81], Metroticket [82] and others [83]. Recent AASLD and EASL guidelines have not yet endorsed Milan expansion and both societies express concern that listing of patients using expanded criteria will cause reduction in survival rates.

Listing of HCC patients for transplantation required adjustments of current prioritization models. The most widely used model for predicting short-term mortality in liver disease is the *model for end stage liver disease* (MELD). The MELD was designed to predict mortality in persons with alcoholic liver disease undergoing a transjugular intrahepatic portosystemic shunt (TIPS) procedure. MELD uses the patients' values for serum bilirubin, serum creatinine, and the international normalized ratio for prothrombin time (INR) to predict 90-day mortality, and scores range from 6 (less ill) to 40 (gravely ill). The score is now widely used for allocation of organs for liver transplantation; however, it cannot predict mortality in HCC [84]. Patients with HCC and compensated liver disease typically have low calculated MELD scores, and may thus dropout from the waiting list or die from tumor progression prior to reaching liver transplantation. To address this waiting list dropout, the United Network of Organ Sharing (UNOS) and Organ Procurement and Transplantation Network (OPTN) policy allocate priority points for candidates with HCC that are within the Milan criteria, equating the risk of tumor progression with the risk of death in patients with chronic liver disease without HCC [85]. Patients with HCC meeting Milan criteria are allocated 22 MELD points and the score increases in a stepwise fashion (equivalent to additional 10 % increase in candidate mortality). Repeated imaging is performed to ensure that tumors do not progress beyond the Milan criteria and that no macrovascular invasion or metastases has occurred.

27.5 Role of Liver Biopsy

Pathological diagnosis of HCC is based on the definitions of the International Consensus Group for Hepatocellular Neoplasia [86] and is recommended for all nodules occurring in non-cirrhotic livers, and for those cases with inconclusive or atypical imaging appearance in cirrhotic livers. Sensitivity of liver biopsy depends upon location, size, and expertise, and might range between 70 and 90 % for all tumor sizes. In patients with negative biopsy findings, HCC cannot be definitely ruled out, despite the high negative predictive values (NPV) (up to 90 % in some studies) [87].

HCC typically form masses with a heterogeneous macroscopic appearance, with foci of hemorrhage or necrosis. There are three main histological patterns: *Trabecular pattern*—tumor hepatocytes arranged in plates and separated by sinusoid vascular spaces. *Acinar pattern* showing dilatation of the canaliculi between tumor cells and *solid pattern* composed of thick trabeculae compressed into a compact mass [88].

On cytology, tumor hepatocytes are polygonal, with an eosinophilic granular cytoplasm, rounded nuclei and prominent nucleoli [88].

27.5.1 Importance of Liver Biopsy

Several biopsy procedures have been developed to obtain an adequate tissue sample; these include image-guided, blind or US guided percutaneous needle core biopsy, and transjugular needle core biopsy.

Optimal biopsy size is debatable. For the evaluation of cirrhosis and for staging and grading viral hepatitis a long and wide biopsy specimen is desired (an ideal size is 3 cm long after formalin fixation obtained with a 16 gage needle). Diagnosis of malignancy can be made with narrower (smaller than 18 gage) biopsy needle [89].

Patients with inconclusive biopsy results should undergo enhanced surveillance or a second liver biopsy. Rates of false negative results are higher in patients with nodules located in the posterior and superior segments of the liver (segments 4b, 7, and 8) [90].

Results of a pre-transplantation biopsy may help address the important issue of tumor differentiation and vascular invasion. There is growing evidence that tumor grade has a marked effect on survival after both resection and liver transplantation [91]. The risk of recurrence is higher in patients with moderately or poorly differentiated tumors compared with those with well-differentiated tumors. Despite their possible effect on transplantation, preoperative biopsies are not common in clinical practice.

27.5.2 Pitfalls of Liver Biopsy

Although considered the gold standard for diagnosis, biopsies of small lesions (<2 cm) may not be reliable. When the lesion is small, needle placement may be difficult and one cannot be certain that the sample did indeed originate from the lesion.

Another common problem is dysplastic nodules that are a common finding in cirrhotic livers. High-grade dysplastic nodules bear a high risk for advancing to HCC. These nodules may show unpaired arteries and a reduced portal supply on imaging studies. Unfortunately, even with a needle biopsy, the hallmark features that distinguish a high-grade dysplastic nodule from HCC, namely stromal

invasion, may not be detected [47]. A study comparing contrast-enhanced CT to US guided liver biopsy for dysplastic nodules in cirrhosis showed that diagnosis of HCC occurred more frequently in high-grade than low-grade dysplastic nodules (32.2 % vs. 9.3 % per year) and non contrast-CT pattern predicted neoplastic transformation of dysplastic nodules [92].

In addition to morphological features, several histological characteristics help distinguish HCC from dysplastic tissue. These include positive stains for glypican 3 [93], heat shock protein (HSP) 70, and glutamine synthetase [94]. A recent study prospectively validated the above panel, showing a slight increase in the diagnostic accuracy in an expert setting [95]. Staining for vascular endothelium with CD34 is strongly positive in HCC; this is because unpaired arteries are more clearly identified, whereas in benign tissue the sinusoidal epithelium stains only weakly with this antibody. Cytokeratin stains for biliary epithelium (CK 7 and CK 19) should be negative, and a positive biliary cytokeratin stain makes HCC less likely [96]. Given the difficulty of making a positive diagnosis in tissue from small lesions, the AASLD recommends that pathologists use the full panel of stains listed above to help distinguish high-grade dysplastic nodules from HCC.

Percutaneous biopsy of HCC carries a potential risk of tumor seeding along the needle tract. 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 recipient's own liver has been removed. A systematic review showed that the risk of tumor seeding was 2.7 % (0–11 %) and the median time between biopsy and tumor appearance was 17 month [97, 98]. 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 (subcapsular), 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 [99]. However, increased risk has not been confirmed by another larger study that involved 1314 patients undergoing radiofrequency ablation [100]. Until now, there has not been clear evidence that pre-transplantation biopsy increases the risk of post-transplantation recurrence, independent of needle tract seeding.

27.6 Primary and Secondary Chemoprevention of HCC

The prognosis of HCC is very poor if diagnosed in the symptomatic stage with most studies reporting a 5-year survival of less than 5 %. Primary HCC prevention includes universal vaccination for HBV, antiviral therapy of patients with chronic HBV or HCV, weight reduction, decreased alcohol consumption, minimizing food contamination with aflatoxins, etc.

For patients with genetic diseases such as pre-cirrhotic 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 nonsurgical HCC treatment (secondary prevention) is also of paramount importance and can significantly improve disease-free and overall patient survival.

27.6.1 Primary Prevention

Chronic infection with HBV is the most common global cause of HCC, affecting more than 350 million individuals (6 % of world population) [101]. Thus, vaccination against HBV is the most efficient primary prevention measure currently available to reduce HCC incidence and mortality globally [102]. Population-based universal infant vaccination for HBV has been shown to be effective in preventing neonatal HBV infection from infected mothers (vertical transmission) [103]. The world's first nationwide HBV universal vaccination program for infants was launched in Taiwan in 1984. Seroprevalence of hepatitis B surface antigen (HBsAg) declined from 9.8 % (pre-vaccination period) to 0.6 % in children in Taiwan in the next 20 years following program initiation. In line with the decrease of chronic HBV infection, the incidence of HCC also decreased from 0.52/100,000 for those born between 1974 and 1984 to 0.13 for those born between 1984 and 1986 [104].

A similar program was launched in Alaska that had the highest rates of acute and chronic HBV and HCC in the United States. Since its initiation, the incidence of HCC in persons <20 years decreased from 3/100,000 in 1984–1988 to zero in 1995–1999 and no cases have occurred since 1999 [105]. The Qidong HBV Intervention Study from China demonstrated similar results [106].

In HCV, primary prevention of new infections should be the goal by rigorous implementation of infection control practices to prevent nosocomial and iatrogenic HCV transmission and prevention of HCV person-to-person transmission through counseling and needle exchange programs [107]. Screening all "high risk" individuals for HCV infection is now recommended in many countries. These populations include persons that received blood donation prior to

initiation of blood bank screening, and persons that have ever used intravenous drugs.

The risk of HCC decreases after cessation of alcohol use by 6–10 % a year; after several years the risk becomes equal to that of the general population [108]. Elimination of aflatoxin (AFB_1) from the food supply in areas where agricultural products are stored under conditions that favor the growth of Aspergillus flavus and Aspergillus parasiticus is also needed [101]. AFB_1 exhibits remarkable synergistic hepatocarcinogenic effect with HBV [109]. The risk of liver cancer in individuals exposed to chronic HBV infection and aflatoxin is up to 30 times greater than the risk in individuals exposed to aflatoxin alone [109].

27.6.2 Treatment of HBV and Prevention of HCC

While both HBV and HCV are linked to HCC, risk of cancer differs between the two viruses. About 10 % of HBV-associated cancers occur in patients without cirrhosis (dependent on selection criteria), whereas HCC mostly occurs in the presence of cirrhosis in HCV infection. HCC risk is increased in those who are hepatitis B e antigen (HBeAg) positive or have detectable HBV DNA. Data from a population-based prospective cohort study of more than 3500 patients from Taiwan, has shown that the progression to cirrhosis in HBV-infected patients is correlated strongly with the level of HBV DNA [110] and that elevated HBV DNA level (>10,000 copies/mL) is a strong predictor of HCC development, independent of HBeAg, serum alanine aminotransferase level, and liver cirrhosis [111]. The risk of HCC increases with the level of HBV DNA inferring that suppression of viral replication with antiviral therapy may decrease the risk of cancer. Two therapeutic approaches for viral suppression are available for patients infected with HBV: Pegylated interferon provides patients with a finite duration of treatment. However, the drug is associated with multiple side effects. Characteristics indicating a favorable response to interferon include low HBV DNA levels, high levels of ALT, presence of the HBV genotype A or B, and lack of advanced liver disease [112]. The other approach is use of oral nuclos(t)ide analogs. These agents are usually given for prolonged periods, but require a single, once daily administration and have a favorable safety profile with minimal side effects. International guidelines currently recommend the use of the newer nuclos(t)ide analogs, entecavir and tenofovir as first line therapy for patients with HBV because of their high resistance barrier. Their use is recommended depending on viral load, HBeAg status, level of hepatic inflammation and clinical condition (i.e., decompensated cirrhosis).

A series of studies suggested that interferon therapy may decrease the risk of HCC development [113–115]. It was later also shown that nucleos(t)ide analog treatment reduces the incidence of HCC. Lamivudine, a first generation nuclostide, reduced the risk of HBV-related HCC from 7.4 to 3.9 % (hazard ratio 0.49) in a prospective trial enrolling 651 Taiwanese patients, and from 13.3 to 1.1 % in a retrospective survey of 2795 Japanese patients [116, 117]. This strongly suggests that there is a significant benefit of viral suppression in reducing risk of HCC development in patients with chronic HBV infection.

27.6.3 Treatment of HCV and Prevention of HCC

Development of HCC occurs almost exclusively in the setting of cirrhosis with HCV infection. In the HALT-C study [6], the 5-year risk of patients with bridging fibrosis and cirrhosis to develop HCC was 5.0 %. The study assessed viral suppression in patients that did not achieve sustained virological response (SVR). 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 [118]. However, in subgroup analysis of long-term HALT-C follow-up, patients with cirrhosis at baseline who were assigned to treatment did have a lower incidence of HCC [119].

A later meta-analysis by Morgan clearly showed that achieving SVR in HCV-infected patient is associated with a marked relative risk (RR) reduction for HCC (RR 0.23, 95 %CI 0.18–0.31) [120].

Another recent review by van der Meer et al. [121] clearly shows that eradication of HCV is associated with regression of liver fibrosis, reduction of portal pressure and lower risk of HCC and liver failure. SVR has been repeatedly associated with improvements in health-related quality of life, hepatic inflammation and fibrosis, and reduction in portal pressure, as well as with a reduced occurrence of solid clinical endpoints such as HCC, liver failure and death. Collectively, this strongly argues that SVR is a patient-relevant endpoint and reasonably likely to predict clinical benefit.

Even with HCV eradication, the initiation of carcinogenic mechanisms has likely occurred many years before viral clearance, and so the threat of HCC remains even if fibrosis decreases. Therefore, patients with advanced fibrosis/cirrhosis who clear HCV should remain under surveillance. Patients that eradicated the virus prior to developing cirrhosis have a very low likelihood of developing HCC and probably do not warrant surveillance [120].

27.6.4 Secondary Prevention

Recent studies have shown marked benefits of HBV or HCV antiviral therapy in reducing the risk of recurrent HCC after initial therapy (resection, transplantation, or ablative therapies). A Japanese study comparing 29 patients with primary HCV-related HCC (stage I/II), receiving combination therapy with PEG-IFN α-2b and RBV after treatment of HCC (hepatic resection, transplantation, RFA, and TACE), to 25 patient who did not receive treatment, has shown 1- and 3-year cumulative survival rates of 100.0 and 90.2 % in the treatment group, and 96.0 and 61.2 % in the non treatment group, respectively [122]. A Taiwanese study demonstrated reduction in HBV-related post resection HCC recurrence in patients treated with nucleoside analogs [123]. As of 2015 there are no consensus recommendations to treat chronic HCV/HBV infection in treated HCC patients to prevent recurrence. However, as the data on recurrence risk reduction accumulates, it seems reasonable to start antiviral treatment for patients undergoing curative intervention for HCC.

27.7 Conclusions

HCC is a deadly cancer occurring on the background of chronic liver disease. In this chapter, we discussed the pivotal role clinicians play in recognizing the various clinical manifestations of HCC; in meticulously screening the population at risk; and in directing the further evaluation of patients with positive diagnostic findings. Early diagnosis represents a major prognostic benefit. There is strong evidence to support screening and there is a well-defined population at risk and low cost, noninvasive, and effective diagnostic tools. It is of paramount importance to diagnose patients with HCC at a stage where a curative approach can still be adopted rather than one of palliation.

The clinician also plays a crucial role in preventing this malignancy. The most important step is primary prevention, through vaccination, dietary, and life style modifications and other treatments of the underlying liver disease. For patients diagnosed with early HCC curative treatments exist which can provide excellent long-term survival.

Patients with HCC are unique, because they combine the complexity of the underlying liver disease and that of the malignancy. Only through better knowledge of the underlying mechanisms in both hepatology and oncology, will we be able to improve survival in patients with this deadly malignancy.

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Current HCC Staging Systems: Their Uses and Limitations

28

Yukio Tokumitsu and Hiroaki Nagano

Contents

28.1 Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer, the third most common cause for cancer death in the world [1]. Because of the high recurrence rate and poor prognosis, the prognostic assessment and selection of treatment strategy in HCC patients are quite important [1–3], and a precise stratification system for the prognosis of HCC patients is required.

In patients with HCC, the prediction of prognosis is complex compared with most solid tumors. It is well known that the prognosis and treatment of HCC depend on the tumor burden in addition to patient's underlying liver disease and liver functional reserve [4, 5]. However, the latter is not integrated in the tumor lymph node metastasis (TNM) staging system, which is generally accepted as a standard approach for prognostication in many cancer clinical staging systems. Therefore, staging systems based on information regarding both tumor factors and host factors such as liver function have been required to accurately classify HCC patients undergoing various therapeutic options [4–7].

An accurate staging system could contribute to prognostication, guiding management decision, comparing different treatment modalities, and comparing treatment outcomes among different institutions [4]. Nowadays, many staging and scoring systems based on both tumor factors and host factors have been proposed for the classification and prognosis of patients with HCC [6–9].

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However, there is no consensus on which is the best prognostic staging system for HCC until now, because there is considerable geographic and institutional variation in both risk factors attributable to the underlying liver diseases and the management of HCC. For example, most of HBV-related HCC patients are particularly prevalent in Africa and Asia, in contrast, most of HCV-related HCC patients are prevalent in western countries, Taiwan and Japan [10, 11]. Other strong risk factors exist, such as alcohol, metabolic syndrome. The characteristics of HCC and screening program which can increase the chance of curative treatment and improve survival also vary with geographic location.

The aim of this review is to focus on the currently available staging systems which integrated tumor factors and host factors for assessing the prognosis of HCC, their uses and limitations.

28.2 Staging Systems of HCC

Generally, the TNM staging that include the extension of the tumor burden in the original primary organ and its spread throughout the body is exhaustive for most solid tumors. Currently, the TNM staging which proposed from the Liver Cancer Study Group of Japan (LCSGJ) and from the AJCC/International Union Against Cancer (UICC) are available for HCC [12–14]. Both of them were developed based on the analysis of patients who received hepatic resection. In 1983, the LCSGJ first introduced an HCC Tumor Node Metastasis (TNM) scheme, which has subsequently been revised, most recently from 5th to the 6th edition in 2015. On the other hand, Vauthey et al. [15] developed a simplified staging system for HCC in 2002, which was adopted as the TNM staging system of AJCC/UICC after minor changes. It has been revised and now, 7th edition was available. These 2 staging systems have some similarities; for example, patients with distant metastasis are assigned to the highest stage, and those with hepatic lymph node metastasis are assigned to the second highest stage. In contrast, the major differences between LCSGJ TNM and AJCC/UICC TNM are the cutoff value for tumor size and its application in prognostic classification [14].

Both the LCSGJ-stage and the AJCC-stage were developed based on a survival analysis of patients who underwent hepatic resection. Although these TNM staging systems are appropriate for patients who will undergo hepatic resection, however, many authors have noted that TNM staging dose not accurately predict outcome for HCC patients undergoing various therapeutic options, because it does not consider liver function status [9].

Thus, nowadays, many staging and scoring systems based on both tumor factors and host factors such as liver function have been proposed for the classification and prognosis of patients with HCC (Table 28.1). In this review, these staging systems are conveniently divided into four categories.

1. Conventional staging systems

These were very famous and pioneering staging systems which attempted to combine tumor factor and liver function, however, not suitable at the present day. Okuda staging and the Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire (GRETCH) staging belong to this category. These often made way to the development of more accurate staging systems and functions as the standard for comparison.

2. Staging systems for treatable condition

The staging systems classified into this category are considered to be suitable for estimating the prognosis of HCC patients who are in treatable condition such as surgery or other locoregional therapy. In this category, the Cancer of the Liver Italian Program (CLIP) score and the Japan Integrated Staging (JIS) score are well-known staging systems, and many staging and scoring systems have been proposed for the classification and prognosis of these population.

3. Staging systems for advanced condition

These staging systems are applicable for advanced HCC who were not amendable to surgery or locoregional therapy. Chinese University Prognostic Index (CUPI) and Advanced Liver Cancer Prognostic System (ALCPS) belong to this category. The advent of effective systemic treatment options are needed for this population with such advanced HCC.

4. Staging systems for treatment recommendation

These staging systems provide treatment algorisms. The Barcelona clinic liver cancer (BCLC) staging is well known and provides treatment algorisms and recommendations, and the prognostic value has been externally validated in many countries. Very recently, the Hong Kong Liver Cancer (HKLC) classification was constructed to developed treatment guidance for Asian patients.

These categories and components of each staging system are showed in Table 28.2.

28.3 Statistical Approach for Comparison of the Staging Systems

To compare the prognostic ability of each staging system with different numbers of parameters, statistical analyses were used in many literatures. The area under the receiver

Table 28.1 Current HCC staging systems

Model	Author	Country	Year	Case number	Patient population	Treatment modality
						Curative[a]/noncurative[b]/palliative
Okuda	Okuda [85]	Japan	1985	850	All	157/464/229
CLIP	CLIP investigators [49]	Italy	1998	435	All	150/97/182 (6 cases unknown)
GRETCH	Chevret [48]	France	1999	761	All	83/277/401
BCLC	Llovet [80]	Spain	1999	[c]	All	–
CUPI	Leung [78]	China	2002	926	All	96 (surgical)/289 (non surgical)/ 541
JIS	Kudo [57]	Japan	2003	722	All	n.d.
JIS family						
Modified JIS	Nanashima [60]	Japan	2004	101	Surgery	101/0/0
SLIDE	Omagari [64]	Japan	2004	177	All	71/92/14
bm-JIS	Kitail [66]	Japan	2008	1924	All	892/934/98
Tokyo	Tateishi [67]	Japan	2005	403	Radiotherapy, surgery	403/0/0
BALAD	Toyoda [74]	Japan	2006	2600	All	1473/959/168
ALCPS	Yau [79]	China	2008	1470	Advanced	0/632/838
TIS	Hsu [68]	Taiwan	2010	2030	All	927/769/334
HKLC	Yau [85]	China	2014	3856	All	1489/1611/756
MITS	Tokumitsu [77]	Japan	2015	234	Surgery	234/0/0

CLIP The Cancer of the Liver Italian Program, *GRETCH* The Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire, *BCLC* The Barcelona Clinic Liver Cancer, *CUPI* Chinese University Prognostic Index, *JIS* The Japan Integrated Staging, *bm-JIS* biomarker-JIS, *ALCPS* Advanced Liver Cancer Prognostic System, *TIS* The Taipei Integrated Score, *HKLC* The Hong Kong Liver Cancer, *MITS* The Mathematical Integrated model for Tumor Staging, *n.d* not described

[a]Curative: surgical resection, liver transplantation and local ablation

[b]Noncurative: transarterial therapy, Radiation therapy, and systemic therapy such as Sorafenib

[c]Derived from the results of a study of the outcomes of radical therapy and/or the natural history of untreated HCC patients

operating characteristic curve (AUC) [16–22], linear trend chi-square score [17, 21, 23–29], likelihood ratio chi-square score [8, 17, 23–36], and Akaike information criteria within a Cox proportional hazards regression model were used to compare the predictive ability of each staging system in many literatures [8, 22–26, 28–45]. Recently, Harrell's C-index was also used in several reports [22, 32, 39, 40, 43, 46].

28.4 Conventional Staging Systems

28.4.1 Okuda Staging System (Table 28.3)

The staging system proposed by Okuda et al. (Okuda) in 1985 is the first attempt to successfully combine the anatomical features of the tumor to the degree of the underlining liver disease [47]. It incorporates the tumor size ($\leq$ or >50 % of the entire liver), presence or absence of ascites, serum albumin level ($\leq$ or >3.0 g/dL), and serum bilirubin level ($\leq$ or >3.0 mg/dL), in which patients are classified into three stages based on these variables. Although the Okuda system was the first integrated system for classifying HCC patients, tumor burden which is evaluated by only tumor extension ($\leq$ or >50 % of the entire liver) was too rough, considering recent developments in imaging modality and the use of adequate surveillance programs. Therefore, the Okuda system often makes way to the development of more accurate staging systems and functions as the standard for comparison.

28.4.2 The Groupe D'Etude et de Traitement Du Carcinome Hépatocellulaire (GRETCH) System (Table 28.4)

The GRETCH system was proposed by the French group Goupe d'Etude et de in 1999 [48]. This system is derived from the finding of a prospective cohort of 761 HCC patients (516 training cohort, 255 validation cohort) treated at 24 Western medical centers. On the basis of a multivariate Cox model in validation cohort, five prognostic factors were

Table 28.2 The categories and components of each staging system

Variables	Conventional staging		Staging for treatable condition									Staging for advanced condition		Staging for treatment recommendation	
	Okuda	GRETCH	CLIP	JIS	JIS family			Tokyo	TIS	MITS	BALAD	CUPI	ALCPS	BCLC	HLKC
					mJIS	SLIDE	bmJIS								
Bilirubin	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Albumin	•		•	•	•	•	•	•	•	•	•			•	•
ICGR15					•	•				•					
ALP		•										•	•		
Portal hypertension														•	
Ascites	•		•	•	•	•	•		•	•		•	•	•	•
Presence of symptoms and/or general status		•										•	•	•	•
Child-Pugh score			•	•			•		•				•	•	•
Liver damage score					•	•				•					
Tumor size	•		•	•	•	•	•	•	•	•		•	•	•	•
Morphological features			•											•	
Numbers of nodules				•	•	•	•	•	•	•		•		•	•
Vascular invasion[a]				•	•	•	•					•			
Portal vein thrombosis		•	•	•	•	•	•					•	•	•	•
Distant metastasis				•	•	•	•					•	•	•	•
TNM				•	•	•	•					•			
AFP		•	•				•		•		•		•		
AFP-L3							•				•				
DCP						•	•				•				

ALP alkaline phosphatase, *ICGR15* indocyanine green retention rate at 15 min, *TNM* tumor node metastasis, *AFP* alpha-fetoprotein, *AFP-L3* lens culinaris agglutinin-reactive alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin

[a]Involving any branch of portal or hepatic vein

Table 28.3 Okuda staging system

	Score	
	0	1
Tumor size	≤ 50 % of the liver	>50 % of the liver
Albumin (g/dL)	≥ 3	<3
Bilirubin (mg/dL)	<3	≥ 3
Ascites	Absent	Present

Table 28.4 GRETCH score

	Score			
	0	1	2	3
Karnofsky index	≥ 80 %			<80 %
Bilirubin (μmol/L)	<50			≥ 50
ALP	<2 × ULN		≥ 2 × ULN	
AFP (μg/L)	<35		≥ 35	
Portal vein thrombosis	Absent		Present	

GRETCH The Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire, *ALP* alkaline phosphatase, *AFP* alpha-fetoprotein

selected: Karnofsky index, serum bilirubin, serum alkaline phosphatase, serum alpha-fetoprotein, and ultrasonographic evidence of portal obstruction. Patients are classified into three risk groups according to these factors, and the author reported that the overall survival differs markedly for the three groups in both training and validation cohort. However, half of the patients (401/761, 53 %) in this study received no specific therapy, therefore, this score may not be suitable for predicting the survival of HCC patients nowadays, considering recent developments in treatment modality and the use of adequate surveillance programs. Thus, it is not a well validated or a widely used staging system.

28.5 Staging Systems for Treatable Condition

28.5.1 The Cancer of the Liver Italian Program (CLIP) Score (Table 28.5)

The CLIP score was derived in 1998 from a retrospective evaluation of 435 Italian patients with HCC treated at 16 Italian institutions [49] for the purpose of producing a more sensitive prognostic index than the Okuda staging system. It includes four variables as the Child-Pugh stage, Tumor morphology, AFP level, Portal vein thrombosis. Subsequently, the same group externally validated the CLIP score in 196 HCC patients enrolled in a randomized clinical trial and confirmed the greater predictive accuracy of this score compared with the Okuda staging system [50]. After that, the CLIP score was developed using an appropriate method and has been externally validated over the world [16–18, 30, 37–41, 51, 52]. It is generally accepted that the CLIP score is suitable for use in HCC patients with intermediate-advanced tumors or those receiving non-surgical treatments. In fact, investigators from Korea [53], Canada [51], Italy [17, 30], France [37], Taiwan [38, 52], the United States [54], and Germany [39] recently demonstrated that the CLIP score provides better prognostic value than other staging systems in HCC patients who received specific treatment modalities, including TACE or radioembolization, systemic chemotherapy with intermediate-advanced tumors. Although studies from Japan [18] and Taiwan [16] have shown that the CLIP score provides a superior predictive value compared to other staging systems for HCC patients undergoing surgical resection, however, there were HCC patients with large size advanced tumor or those receiving major hepatectomy. Therefore, this score may not be suitable for predicting the survival of the early stage HCC, which are susceptible to percutaneous or minor hepatectomy.

28.5.2 CLIP Family

Staging systems based on the CLIP score are conveniently classified into "CLIP family" in this review. In recent years, Kaseb et al. [55] proposed the VEGF-CLIP (V-CLIP) score based on the VEGF, which was the major mediator of angiogenesis in the setting of HCC. The authors integrated the VEGF into the CLIP score, and they reported that the V-CLIP score provides superior predictive accuracy compared to the conventional CLIP score. The same group proposed the insulin-like growth factor-1 (IGF-1) CLIP (I-CLIP) [56] score based on findings demonstrating that the IGF-1 value, which reflects the synthetic function of the liver. The authors added the IGF-1 to the CLIP score and created the V-CLIP score. They also reported that the I-CLIP score provides superior predictive accuracy compared to the conventional CLIP score.

28.5.3 The Japan Integrated Staging (JIS) Score (Table 28.6)

Kudo et al. [57] originally proposed the JIS score, which is defined by the LCSGJ TNM stage and the Child-Pugh classification. It is derived from a cohort of 722 HCC patients treated at two Japanese institutions. Patients with a Child-Pugh grade A, B, and C status are allocated a score of 0, 1, and 2, respectively, and patients with the TNM stage by LCSGJ of stage I, II, III, and IV are allocated to score of 0, 1, 2, and 3, respectively. Subsequently, patients are classified

Table 28.5 CLIP score

	Score		
	0	1	2
Tumor morphology	Uninodular and extension ≤50 %	Multinodular and extension ≤50 %	Massive or extension >50 %
Child-Pugh classification	A	B	C
AFP (ng/mL)	<400	≥400	
Portal vein thrombosis	Absent	Present	

CLIP The Cancer of the Liver Italian Program, *AFP* alpha-fetoprotein

Table 28.6 JIS score

	Score			
	0	1	2	3
TNM stage by LCSGJ	I	II	III	IV
Child-Pugh classification	A	B	C	

JIS The Japan Integrated Staging, *TNM* tumor node metastasis, *LCSGJ* Liver Cancer Study Group of Japan

into six groups (0–5) based on the sum of these scores. Using 4525 patients with HCC at five institutions, the same group validated the JIS score as a good prognostic staging system than the CLIP score [31]. Other studies from Japan have also demonstrated that the JIS score to be the best prognostic model in HCC patients who receive various treatment modalities [8, 16, 32, 44]. Toyoda et al. [44] showed that the JIS system was the most suitable after 1990, when early detection and early treatment of HCC became common, although the CLIP staging systems proved to be more suitable before 1991. After 1990, surveillance of patients at high risk for development of HCC caused by chronic viral hepatitis or cirrhosis and early detection of HCC were very common in Japan, because of development of various scanning modality as well as indication of highly sensitive tumor markers [58, 59]. The discriminating power of JIS system is, therefore, particularly suitable for countries such as Japan, where many small HCC are detected and diagnosed at early stages and treated with radical therapies. However, it has not been well validated in countries outside of Japan, especially in a western patient population.

28.5.4 JIS Family (Table 28.7)

Integrated staging systems based on the Japanese TNM stage by LCSCJ are conveniently classified into "JIS family" in this review.

28.5.4.1 Modified JIS Score

Nanashima et al. [60] proposed m-JIS score, which combined TNM staging system by LCSGJ and the degree of the liver damage (Table 28.8) instead of Child-Pugh classification, and reported that this system was better predictor of prognosis than JIS score in HCC patients who underwent hepatic resection [45]. Ikai et al. [61] validated this system using the records of 42,269 patients diagnosed with HCC that were registered between 1992 and 1999 in a nationwide Japanese database. This suggested that the degree of liver

Table 28.7 JIS family

	Score			
	0	1	2	3
Modified JIS score				
TNM stage by LCSGJ	I	II	III	IV
Liver damage classification	A	B	C	
SLiDe score				
TNM stage by LCSGJ	I	II	III	IV
Liver damage classification	A	B	C	
DCP (mAu/mL)	<400	≥400		
bm-JIS score				
TNM stage by LCSGJ	I	II	III	IV
Child-Pugh classification	A	B	C	
No of elevated tumor marker (AFP, AFP-L3, DCP)	0	1	2–3	

JIS The Japan Integrated Staging, *TNM* tumor node metastasis, *LCSGJ* Liver Cancer Study Group of Japan, *AFP* alpha-fetoprotein, *AFP-L3* Lens culinaris agglutinin-reactive alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin

Table 28.8 Liver damage classification by LCSGJ

Item	Liver damage grade		
	A	B	C
Ascites	None	Controllable	Uncontrollable
Bilirubin (mg/dL)	<2.0	2.0–3.0	>3.0
Albumin (g/dL)	>3.5	3.0–3.5	<3.0
ICG R15 (%)	<15	15–40	>40
Prothrombin activity (%)	>80	50–80	<50

LCSGJ Liver Cancer Study Group of Japan, *ICGR15* indocyanine green retention rate at 15 min

damage could evaluate and classify liver function more precisely than the Child-Pugh classification for early HCC or surgical population. The degree of liver damage classification was proposed by the LCSGJ, and incorporates the ICGR15 test, which is an estimation of indocyanine green clearance, instead of encephalopathy in the Child-Pugh classification system. ICGR15 test has been widely used in the field of surgery in Japan as a useful marker of hepatic function [62, 63]. However, ICGR15 are not routinely assessed in other parts of the world, thus, the m-JIS score has not been validated in countries outside of Japan.

28.5.4.2 SLiDe Score

Omagari et al. [64] proposed SLiDe score, which combined TNM staging system by LCSGJ, the degree of the Liver damage and DCP (SLiDe). They showed that there was clear discrimination among the survival curves plotted for patients with different SLiDe scores, and this system could predict the outcome of HCC patients more precisely than the CLIP and JIS scoring systems in these population. Nanashima et al. [65] validated this system in 207 HCC patients who undergone hepatic resection. However, SLiDe score does not seem to be very suitable for worldwide use at present, because it uses some parameters that are not routinely assessed in other parts of the world such as ICGR15 test and DCP. Therefore, this classification should be further validated in other large study populations.

28.5.4.3 Biomarker-JIS Score

The JIS staging classification was further modified by Kitai et al. [66]. They proposed biomarker-combined JIS (bm-JIS) which combined TNM staging system by LCSGJ, the Child-Pugh classification, and three tumor markers for HCC, namely AFP, lens culinaris agglutinin-reactive AFP (AFP-L3), and des carboxyprothrombin (DCP). They validated the bm-JIS score as a good prognostic staging system than the conventional JIS sore [33, 34, 66], BALAD score [33], and BCLC system [34]. Although this scoring system validated in a relatively large population of HCC patients in Japan, this system has now been externally validated from but still requires validation in a western patient population, because measuring all of these three tumor markers in routine clinical practice are uncommon worldwide.

Table 28.9 Tokyo score

	Score		
	0	1	2
Albumin (g/dL)	>3.5	2.8–3.5	<2.8
Bilirubin (mg/dL)	<1	1–2	>2
Tumor size (cm)	<2	2–5	>5
Number of nodules	≤ 3	–	>3

28.5.5 TOKYO Score (Table 28.9)

Tateishi et al. [67] proposed the Tokyo score would provide a prediction of prognosis for patients who were candidates for radical therapy, such as percutaneous ablation or surgical resection. A total of 403 patients with HCC treated by percutaneous ablation were used as the training sample to develop the Tokyo Score and validated by 203 independent patients who underwent hepatectomy at the same institution and demonstrated that the predictive ability of the Tokyo score is equal to that of the CLIP score and better than that of the BCLC classification.

Investigators from Taiwan [29] reported that the Tokyo score was the most informative staging system in a large cohort (n = 2010) of HCC patients with predominant HBV infection who underwent surgical resection or transarterial chemoembolization. However, the Tokyo score has not been validated in a Western population. Further, external validation of the Tokyo classification in different patient populations is needed.

28.5.6 The Taipei Integrated Score (TIS) System (Table 28.10)

The Taipei Integrated Score System (TIS) was proposed by Hsu et al. [68] in 2010. This system is derived from the investigation of a cohort of 2030 HCC patients undergoing

Table 28.10 TIS

Variable	Score			
	0	1	2	3
Total tumor volume (cm^3)	<50	50–250	250–500	>500
Child-Pugh classification	A	B	C	
AFP (ng/nL)	≤ 400	>400		

TIS Taipei integrated system, *AFP* Alpha-fetoprotein

different treatment modalities at a single institution in Taiwan. The authors adopted the calculated total tumor volume (TTV) as a surrogate marker of the tumor burden. TTV was defined as the sum of the volume of each tumor [(4/3) × 3.14 × (radius of tumor in cm)^3]. Subsequently, they combined the TTV with four cirrhosis-associated models (Child-Pugh grade, MELD, MELDNa and MELD-Na) and/or tumor factors (serum AFP levels and vascular invasion) to create the TTV-based staging system and the prognostic ability of the TTV-based staging system and the four current systems, including the BCLC, CLIP, JIS, and Tokyo score was examined. They reported that the TTV-CTP-AFP model [i.e. The Taipei Integrated Score System (TIS)] provided the best prognostic ability among them and the model was validated in Taiwanese population [35, 36]. The TTV and TTV-based staging systems are also evaluated to predict recurrence of HCC after liver transplantation in many countries [69–73]. However, the TTV value may not be accurate in tumors which are not typically spherical, such as infiltrative or numberless type, because the TTV is estimated based on the assumption that all tumors are spherical.

28.5.7 BALAD Score (Table 28.11)

BALAD score was constructed by Toyoda et al. in 2006 for the purpose of providing a simple and objective staging system that requires no imaging studies or pathological or clinical evaluations [74]. There were five variables in the BALAD score: The Bilirubin, Albumin, Lens culinaris agglutinin-reactive alpha-fetoprotein (AFP-L3), AFP, and DCP Score. This score is derived from the findings of a cohort of 2600 HCC patients treated at five Japanese institutions. The authors adopted three tumor markers (AFP-L3 > 15 %, AFP > 400 ng/dL, DCP > 100 mAU/mL) as factors reflecting tumor progression and also used two serum markers (serum bilirubin and albumin) as factors indicating the liver functional reserve. They reported that the discriminative ability of the BALAD score was comparable to that of the CLIP score and JIS score. The BALAD score is a simple and objective tool that requires the use of only a serum sample, without imaging, pathological, or clinical assessments. Although it was considered that measuring the AFP-L3 and DCP values in routine clinical practice worldwide were uncommon, however, this system was externally validated in recent years in countries outside of Japan [75, 76].

Table 28.11 BALAD score

	Score			
	0	1	2	3
Albumin (g/dL)	>3.5	2.8–3.5	<2.8	
Bilirubin (mg/dL)	<1	1–2	>2	
Bilirubin-albumin score*	A	B	C	
No of elevated tumor marker (AFP, AFP-L3, DCP)	0	1	2	3

*Liver function was categorized by the sum of these 2 points (i.e., bilirubin and albumin) as scores A (0–1 points), B (2–3 points), and C (4 points)
AFP alpha-fetoprotein, *AFP-L3* lens culinaris agglutinin-reactive alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin

28.5.8 The Mathematical Integrated Model for Tumor Staging (MITS) Score (Table 28.12)

More recently, we developed a novel predictive system based on mathematical product of tumor number and size of largest tumor ($N \times S$ factor) for prognosis of Japanese HCC patients after hepatectomy [77]. We found that cutoff value of $N \times S$ factor at 4 and 9 had high accuracy in predicting recurrence of HCC. Given that the $N \times S$ factor and the degree of Liver Damage classification by LCSGJ were independent risk factors for HCC prognosis by multivariate analysis, we constructed the mathematical integrated model for tumor staging (MITS) score by combining the $N \times S$ factor with the degree of Liver Damage classification. In this population, we showed that the MITS score was more predictable for the prognosis of HCC patients than any of the six well-known clinical staging systems [TNM (LCSGJ), TNM (UICC), JIS score, modified JIS score, CLIP score, and the Tokyo Score]. We found that the $N \times S$ factor-based staging system had high accuracy in predicting HCC prognosis.

There were several limitations in this study: First, it was a retrospective single-center study that enrolled only patients who underwent curative hepatectomy. Second, HCC patients with invasion of major portal or hepatic vein branch were excluded in this study. Third, MITS score integrates the degree of Liver damage classification which incorporates the

Table 28.12 MITS score

	Score		
	0	1	2
Mathematical product of tumor number and size ($N \times S$ factor)	<4	4–9	>9
Liver damage classification	A	B	

MITS The Mathematical Integrated model for Tumor Staging

ICGR15 test instead of Child-Pugh classification system, and ICGR15 are not routinely assessed in other parts of the world or non-surgical populations even in Japan. In this regard, further studies will be needed to evaluate whether the robustness of the $N \times S$ factor-based staging system which may integrate Child-Pugh classification in predicting prognosis could be maintained in a cohort in which the majority of the subjects were HCC patients who received non-surgical treatment.

28.6 Staging Systems for Advanced Condition

28.6.1 Chinese University Prognostic Index (CUPI)(Table 28.13)

The Chines University Prognostic Index (CUPI) was proposed by a Hong-Kong group in 2002 [78]. This score is derived from the results of a cohort of 926 HCC patients treated at a single Hong-Kong hospital. In that study, 19 potential prognostic factors were evaluated in a multivariate analysis using a Cox regression model among 926 Chinese patients, mostly with HBV-associated HCC. Subsequently, five prognostic factors (total bilirubin, presence of ascites, alkaline phosphatase, alpha fetoprotein, and asymptomatic disease on presentation) were selected and added to the TNM, in order to set up 3 classes of risk with highly significant differences in survival. Moreover, the authors demonstrated that the CUPI system is more discriminant in predicting survival than the conventional TNM staging system, Okuda system, or CLIP score. In this study, the cohort was composed of a large proportion of patients who received only best supportive care (58.4 %, vs. resection 10.4 %). Hence, this system is not preferable for assessing patients who undergo curative treatment and several validation studies were performed in Asian population with advanced stage of HCC [28, 40, 43, 46]. In recent years, Chan et al. [78] reported an international validation of the CUPI. They reported that the CUPI was demonstrated to be optimal for those undergoing palliative treatment in both Eastern and Western HCC patient population, and they concluded that a more precise staging system for early-stage disease patients is required.

Table 28.13 CUPI

Variable		Weight
TNM stage	I and II	−3
	III	−1
	IV	0
Bilirubin (μmon/L)	<34	0
	34–51	3
	≥ 52	4
Ascites		3
AFP (ng/mL)	>500	2
ALP (IU/L)	>200 IU/L	3
Asymptomatic disease on presentation		−4

CUPI Chinese University Prognostic Index, *TNM* tumor node metastasis, *AFP* alpha-fetoprotein, *ALP* alkaline phosphatase

28.6.2 Advanced Liver Cancer Prognostic System (ALCPS) (Table 28.14)

The Advanced Liver Cancer Prognostic System (ALPCS) was constructed by Yau et al. [79] in 2008 for the purpose of creating an optimal staging system for classifying advanced HCC patients who were not amendable to surgery or locoregional therapy. This system was derived from the analysis of a cohort of 1470 advanced HCC patients (1109 training set and 361 validation set) treated at a single center in Hong Kong, and developed using 11 prognostic factors with different weights on basis of a multivariate Cox model. They reported that the ALCPS stratified patients in both training and validation sets to different prognostic groups with significant difference in three-month overall survival.

Table 28.14 ALCPS

Characteristics		Points
Ascites	Yes/no	2/0
Abdominal pain	Yes/no	2/0
Weight loss	Yes/no	2/0
Child-Pugh classification	A/B/C	0/2/5
ALP (IU/L)	>200/ ≤ 200	3/0
Bilirubin (mmol/L)	>50/33–50/ ≤ 33	3 1/0
Urea (mmol/L)	>8.9/ ≤ 8.9	2/0
Portal vein thrombosis	Yes/no	3/0
Tumor size	Diffuse/>5 cm/ ≤ 5 cm	4/3/0
Lung metastasis	Yes/no	3/0
AFP (ng/mL)	>400/ ≤ 400	4/0
Prognosis	Score	3-mo survival rate
Good	0–2/3–6/7–8	>0.81/0.72–0.8/0.66–0.69
Intermediate	9/10–12/13–14/15	0.63/0.51–0.59/0.42–0.47/0.38
Poor	16/17–19/20–22/ ≥ 23	0.33/0.21–0.29/0.1–0.17/< 0.1

ALCPS Advanced liver cancer prognostic system, *ALP* Alkaline phosphatase, *AFP* Alpha-fetoprotein

Moreover, the score showed significantly better predictive power in known three-month survival status than Okuda score and CLIP score in the validation set.

Although investigators from China demonstrated the ALCPS system to be the prognostic model in advanced HCC patients [21, 41], however, this score has not yet been validated in a Western population. In addition, many prognostic factors are included in this system (n = 11), calculating the total score somewhat complicated in daily clinical practice.

28.7 Staging Systems for Treatment Recommendation

28.7.1 The Barcelona Clinic Liver Cancer (BCLC) Staging (Fig. 28.1)

The Barcelona Clinic Liver Cancer (BCLC) classification was first proposed by the Barcelona Clinic Liver Cancer group in 1999 [80]. This staging system includes an integrated assessment of liver disease, tumor extension, and presence of constitutional symptoms. This model is derived from the results of a study of the outcomes of radical therapy and/or the natural history of untreated HCC patients, and might be an appropriate classification system for a patient population evenly distributed among early, intermediate, and advanced stages of the disease. The notable feature of the BCLC system is the assignment of treatment recommendations for each stage based on the best treatment options currently available, and this system has been updated according to the results of investigations that have incorporated strong evidence. The BCLC staging system and treatment allocation is summarized in Fig. 28.1. In 2003, the system incorporated the concept of very early stage (BCLC 0) that included patients with HCC 2 cm with well-preserved liver function [10]. With the description of several cohort studies showing the efficacy of ablation in these patients, the scheme was updated again recognizing ablation as first treatment option. In 2008, the positive results of two randomized controlled trial in advanced HCC, allowed the acknowledgment of sorafenib as the first-line treatment option for stage C (advanced stage) patients [3, 81].

Currently, the BCLC classification is endorsed as the standard system for HCC management by the American Association for the Study of Liver Disease, American Gastroenterology Association, European Association for the

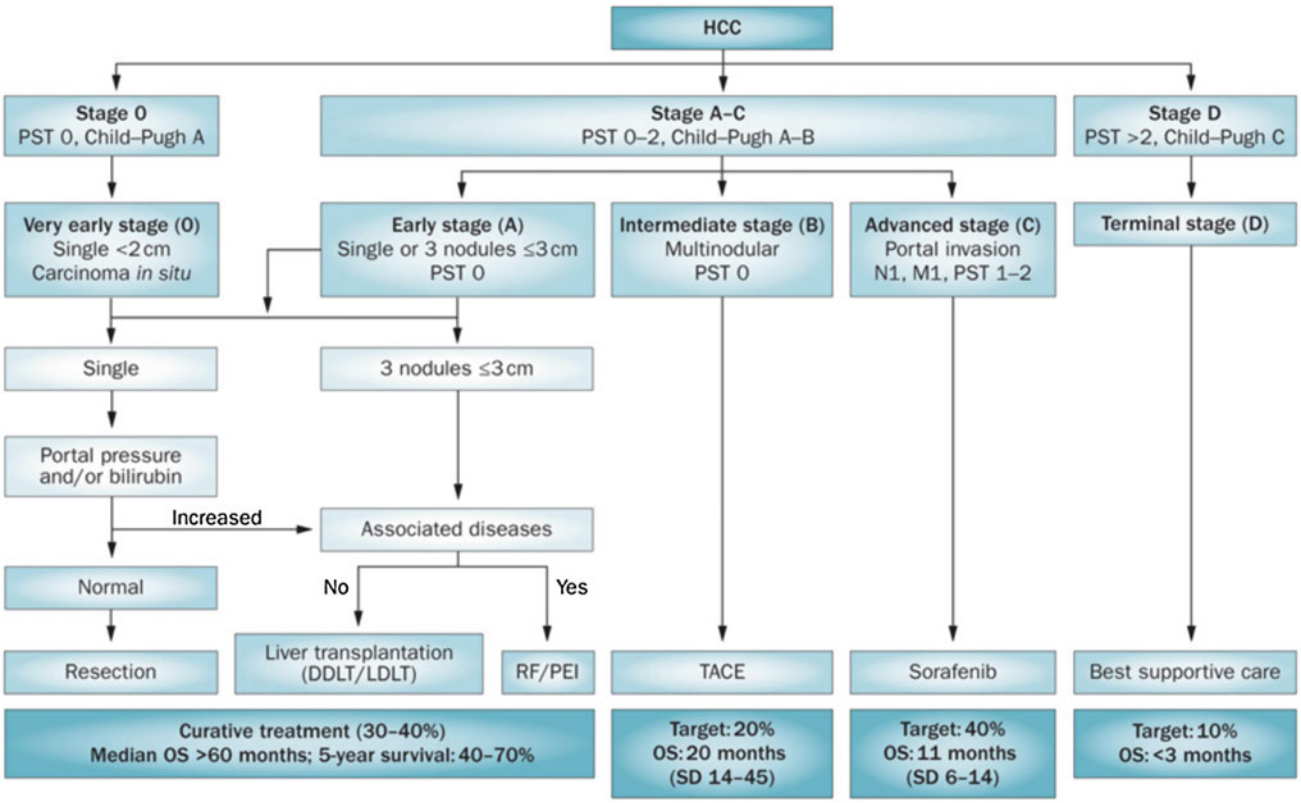

Fig. 28.1 The Barcelona Clinic Liver Cancer (BCLC) staging system for Hepatocellular carcinoma. *M* metastasis classification; *N* node classification; *PST* performance status; *RF* radiofrequency ablation; *PEI* percutaneous Ethanol Injection; *TACE* transarterial chemoembolization. Permission obtained from Elsevier © European Association for the Study of the Liver [88]. Permission from Elsevier

Study of Liver, and the European Organization for the Research and Treatment of Cancer, and it is currently the most used in Western countries [5].

The prognostic value of BCLC staging system has been externally validated in many countries [19, 20, 23–27, 42]. Several investigators from Italy [27] and China [42] have shown that the BCLC classification is the best prognostic model in HCC patients receiving curative therapy. In contrast, studies from Italy [19, 23, 26, 30], the United States [24], Spain [25], South Korea [20] have shown that the BCLC classification provides the best prognostic value in HCC patients with early to advanced stage tumors treated with various modalities. These results indicate that the predictive accuracy of the BCLC classification is highly stable. With regard to treatment allocation, a large-scale trial from Taiwan [82] (n = 3892) showed that the treatment schedules determined according to the BCLC classification are both reasonable and beneficial for survival in patients with HCC.

However, the BCLC classification has some limitations. Although the BCLC treatment schedule recommends that resection be applied only for those very early stage patients without portal hypertension and normal bilirubin levels, however, portal hypertension which is defined as the presence of a hepatic venous pressure gradient >10 mmHg is invasive and not routinely carried out in daily practice worldwide [67]. It might be easier and simpler to use clinical portal hypertension, including esophageal varices or splenomegaly with a platelet count [80]. Indocyanine green retention rate at 15 min as the criteria in selection of the best candidates for resection is also useful [82]. Moreover, BCLC stage B (intermediate stage) includes a considerable heterogeneous population of HCC patients with varying degree of tumor extension, liver functional reserve, and disease etiology, thus resulting in prognostic heterogeneity [83, 84].

28.7.2 The Hong Kong Liver Cancer (HKLC) Staging (Fig. 28.2)

Very recently, the HKLC classification [85] was constructed by a Hong Kong group to developed treatment guidance for Asian patients with HCC. This system is derived from the results of a large cohort of 3856 HCC patients predominantly infected by hepatitis B virus (HBV). ECOG PS, Child-Pugh grade, liver tumor status, and presence of extrahepatic vascular invasion or metastasis were selected while developing the system by using the 1968 training set according to a multivariate analysis. Patients are classified into five main stages and nine substages (stages I–V_b) based on these prognostic factors. Subsequently, the HKLC classification was compared with the BCLC classification in terms of discriminatory ability and effectiveness of treatment recommendation in 1888 test set. They demonstrated that the HKLC system had significantly better ability than the BCLC system to distinguish between patients with specific overall survival times. Notably, the HKLC classification is able to better stratify patients in the BCLC B and C stages into distinct groups, with better survival outcomes based on more aggressive treatment recommendations than that observed in the BCLC treatment algorithm. The HKLC system appears to have a greater impact on the current BCLC classification, addressing the problems with the heterogeneity of the BCLC B and C stages and rigidity of treatment allocation. Yan et al. [22] reported that the HKLC system was more suitable for predicting prognosis in a Chinese cohort of 668 HCC patients than the BCLC classification. External validation in Western population and/or elsewhere is needed.

28.8 Summary of Staging Classifications

It is currently difficult to establish the staging system that is suitable for all patient populations universally. The best staging system to use may differ according to the detection and treatment conditions of HCC. The validation and comparative studies of each staging system are showed in Table 28.3. Each existing staging system may have been characterized by the patient population based on which it was constructed [10]. For example, the incidence of HCC varies considerably with the geographic area because of differences in the major causative factors. Hepatitis B, which is endemic in developing geographic regions such as Eastern Asia and Sub-Saharan Africa, is the main cause of new HCC cases in such areas. Hepatitis C is the predominant cause of HCC in area such as Southern Europe and Japan. In Northern Europe and the USA, HCC is often related to other factors such as alcoholic liver disease. Several studies have shown that HCC patients with HCV infection or alcoholic liver disease exhibit poorer outcomes than those with HBV infection. This is because HCC patients with HBV infection generally have a better liver functional reserve than those with HCV infection or alcoholic liver disease.

Usefulness of the staging systems will differ depending on distribution of HCC stage at diagnosis. For example, CUPI score and ALCPS were suitable staging systems for advanced stages of the disease and validated in a large cohort of HCC patients in China, these are not suitable in country where the early detection and early treatment of HCC are common. In western patient populations, the BCLC staging system appears to be superior based on findings in several studies (two conducted in Italy, one in Taiwan, and one in North America). The JIS score, the JIS family, and the Tokyo score are the suitable staging systems in Japan, where many smaller tumors are detected based on the established screening system for HCC [13, 86]. However, it is the

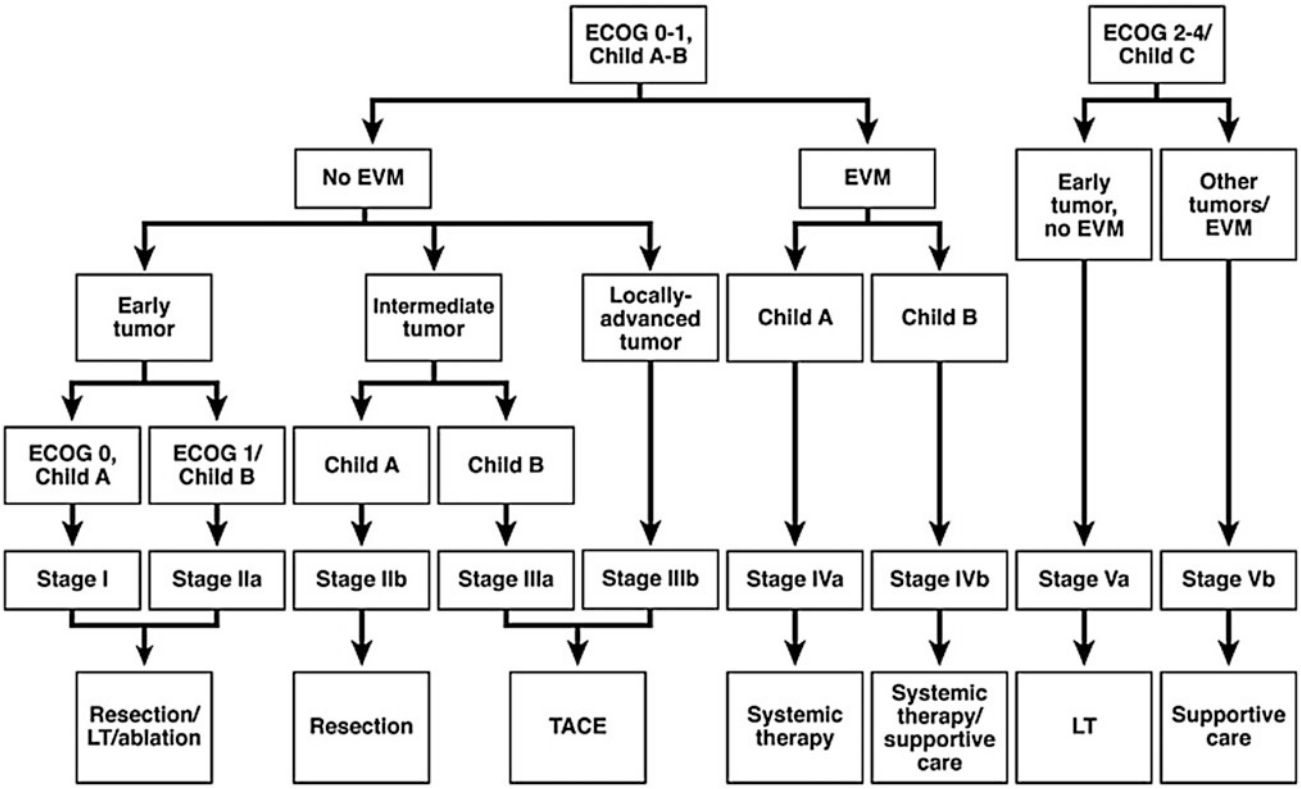

Fig. 28.2 The Hong Kong Liver Cancer (HKLC) prognostic classification scheme. EVM, extrahepatic vascular invasion/metastasis. Early tumor: 5 cm, 3 tumor nodules and no intrahepatic venous invasion; Intermediate tumor: (*1*) 5 cm, either >3 tumor nodules or with intrahepatic venous invasion, or (*2*) >5 cm, 3 tumor nodules and no intrahepatic venous invasion; and Locally advanced tumor: (*1*) 5 cm, >3 tumor nodules and with intrahepatic venous invasion, or (*2*) >5 cm, >3 tumor nodules or/and with intrahepatic venous invasion, or (*3*) diffuse tumor. Modified from Yau et al. [85]. Permission from W. B. Saunders Company

problem that few validation studies of these Japanese staging systems were reported outside Japan (Table 28.15).

Usefulness of the staging systems will also differ depending on the distribution of patients with HCC according to the period. As mentioned above, Toyoda et al. [44] reported that the CLIP staging systems proved to be more suitable before 1990, however, the JIS system was the most suitable after 1990, when early detection and early treatment of HCC became common. When early detection of HCCs becomes more common in many countries, it could lead to the predominance of early-stage HCC patients and Japanese staging systems such as the JIS and the JIS family may become more suitable over the world.

Although the JIS score and JIS family based on the TNM by LCSGJ for HCC were useful in Japan, however, there are some limitations. First, although the Japanese TNM for HCC has been generally accepted as a standard approach for prognostication in Japan, however, it is not always used all over the world. Second, the model included established classifications such as the case for TNM staging can be modified in the future, and different versions may be confused. Third, discrepancies between pre- and postoperative diagnoses in the TNM and the TNM-based staging systems often caused by microvascular invasion detected in resected specimens after hepatectomy. In the first place, the TNM staging was developed based on a survival analysis of surgical patients and their pathological findings, thus, these postoperative histopathological staging systems are appropriate for patients who are scheduled to undergo surgical resection [12, 14]. Although, vascular invasion, one of the TNM staging components, is considered as a prognostic factor, however, peripheral vascular invasion is usually obtained as microvascular invasion in resected specimen and underestimated preoperatively. Thus, pre/postoperative staging discrepancy in the TNM and the TNM-based staging system (the JIS and JIS family) often caused by accompanying newly detected microvascular invasion in the resected liver. In this regard, there is still room for development of novel tumor factor which is simple, robust, and not needed the information on pathological vessel involvement, and the $N \times S$ factor, which consists of mathematical product of tumor number and size of largest tumor, could solve these problems.

One of the goals of staging systems today is to provide an evidence-based treatment guide [80]. All staging

Table 28.15 The validation and comparative studies of each staging system

Suitable model		Country	Year	Case number		Treatment modality	Comparator staging systems
						Cur[a]/Non-cur[b]/Palliative	
CLIP	Levy [51]	Canada	2002	257	ALL	95/29/133	Okuda
	Giannini [30][c]	Italy	2004	81	ALL	25/43/13	Okuda, BCLC, GRETCH
	Chen [16][c]	Taiwan	2007	382	Surgery (major hepatectomy)	382/0/0	Okuda, TNM, BCLC, CUPI, JIS MELD
	Camma [17]	Italy	2008	406	ALL	115/63/228	BCLC, GRETCH
	Collete [37]	French	2008	538	Advanced	0/122/416	Okuda, BCLC
	Cho [53]	Korea	2008	131	TACE	0/131/0	Okuda, BCLC, JIS, Child
	Lin et al. [52]	Taiwan	2009	3668	ALL	662/1768/1438	–
	Noda [18]	Japan	2009	46	Surgery (HCC > 10 cm)	46/0/0	TNM, JIS
	Hsu et al. [38]	Taiwan	2010	1713	ALL	797/655/261	TNM, BCLC, JIS, Tokyo
	Op den Winkel [39]	German	2012	405	ALL	95/263/47	JIS, Okuda, GRETCH, TNM, BCLC, Child
	Shao et al. [40][c]	Taiwan	2012	157	Advanced	0/157/0	GRETEC, CUPI, Okuda, Tokyo, JIS, BCLC, CIS, AJCC
	Lin et al. [41][c]	Taiwan	2012	156	Advanced	0/0/156	TNM, Okuda, CUPI, JIS, Tokyo, ALCPS
	Memon [54]	USA	2014	428	TARE	0/428/0	Okuda, BCLC, GRETCH, CUPI, JIS
GRETCH	Giannini [30][c]	Italy	2004	81	ALL	25/43/13	Okuda, BCLC, CLIP
BCLC	Cillo [23]	Italy	2004	187	ALL	119/40/28	Okuda, CLIP, GRETCH, CUPI
	Giannini [30][c]	Italy	2004	81	ALL	25/43/13	Okuda, CLIP, GRETCH
	Grieco [19]	Italy	2005	268	Early to intermediate	146/103/19	Okuda, CLIP
	Marrero [24]	USA	2005	244	ALL	107/66/71	Okuda, TNM, CLIP, GRETCH, CUPI, JIS
	Pascual [25]	Spain	2006	115	ALL	38/39/38	Okuda, CLIP, BCLC,GRETCH, MELD, Child
	Cillo [26]	Italy	2006	195	ALL	175/9/11	Okuda, CLIP, TNM, JIS,
	Wang [82]	Taiwan	2008	3892	ALL	631/1796/1465	–
	Guglielmi [27]	Italy	2008	112	RFA	112/0/0	Okuda, TNM, CLIP, GRETCH, CUPI, JIS
	Kim [20]	Korea	2012	1717	ALL	357/1188/172	JIS, Tokyo, CLIP, CUPI, GRETCH
	Zhao [42]	China	2015	743	Surgery	743/0/0	TNM, JIS, Tokyo, CLIP, CUPI, Okuda
CUPI	Chan [43]	China	2011	595	ALL	83/206/306	BCLC, CLIP, TNM, Okuda
	Shao [40][c]	Taiwan	2012	157	Advanced	0/157/0	GRETCH, CUPI, Okuda, Tokyo, JIS, BCLC, CIS, AJCC
	Zhang [28]	China	2014	196	Non-surgical treatment	6/114/76	BCLC, CLIP, JIS, CIS, Okuda, TNM
	Chan [46]	China	2014	517	ALL	92/224/201	BCLC, CLIP
	Chan [46]	UK	2014	567	ALL	228/235/104	BCLC, CLIP

(continued)

Table 28.15 (continued)

Suitable model		Country	Year	Case number		Treatment modality	Comparator staging systems
						Cur[a]/Non-cur[b]/Palliative	
JIS	Kudo [31]	Japan	2004	4525	ALL	2023/2306/196	CLIP
	Toyoda [44]	Japan	2005	1508	ALL	598/632/288	CLIP, BCLC
	Kondo [32]	Japan	2007	235	Surgery	235/0/0	CLIP, BCLC, GRETCH, CUPI, mJIS, Tokyo
	Chung [8]	Japan	2008	290	ALL	208/58/24	BCLC, Tokyo
	Chen [16][c]	Taiwan	2007	382	Surgery (minor hepatectomy)	382/0/0	Okuda, CLIP, TNM, BCLC, CUPI, JIS, MELD
m-JIS	Nanashima [45]	Japan	2006	230	Surgery	230/0/0	TNM, JIS CLIP
	Ikai [61]	Japan	2006	42269	ALL	24,421/13,868/3,980	m-CLIP
SLIDE	Nanashima [65]	Japan	2009	207	Surgery	207/0/0	–
bm-JIS	Kitai [33]	Japan	2008	1173	ALL	663/470/36	JIS, BALAD
	Kitai [34]	Japan	2014	4649	ALL	2995/1455/199	JIS, BCLC
Tokyo	Chen [29]	Taiwan	2009	2010	ALL	984/518/478	JIS, CLIP, BCLC, Okuda, TNM
BALAD	Fox [75]	UK	2014	319	ALL	16.1 %/83.9 % (non cur + palliative)	–
	Chan [76]	China	2015	198	ALL	37/87/74	BCLC
ALCPS	Lin [41][c]	Taiwan	2012	156	Advanced	0/0/156	TNM, Okuda, CLIP, CUPI, JIS, Tokyo
	Li [21]	China	2013	208	Advanced	0/10/198	JIS, TNM, CLIP, GRETCH
TIS	Hsu [35]	Taiwan	2012	2203	ALL	1017/1186/0	CLIP, BCLC, JIS
	Chen [36]	Taiwan	2015	467	RFA	467/0/0	BCLC, CLIP, JIS
HKLC	Yan [22]	China	2015	668	ALL	453/205/10	BCLC

RFA radiofrequency ablation, *TACE* transarterial chemoembolization, *TARE* transarterial radioembolization, *TNM* Tumor Node Metastasis, *CLIP* The Cancer of the Liver Italian Program, *GRETCH* The Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire, *BCLC* The Barcelona Clinic Liver Cancer, *CUPI* Chinese University Prognostic Index, *JIS* The Japan Integrated Staging, *bm-JIS* biomarker-JIS, *ALCPS* Advanced Liver Cancer Prognostic System, *TIS* The Taipei Integrated Score, *HKLC* The Hong Kong Liver Cancer, *MELD* Model for End stage Liver Disease, *CIS* China integrated staging

[a]Cur: surgical resection, liver transplantation, and local ablation

[b]Noncur: transarterial therapy, radiation therapy, and systemic therapy such as Sorafenib

[c]The same literature

classifications have been designed to predict prognosis, many staging systems lack optimal treatment allocation except for BCLC and HKLC. However, BCLC treatment recommendations were not suitable in all situations. For example, some prognostic factors, such as the presence of portal hypertension is contraindications, because there are evidences which suggest that hepatic resection can be performed successfully even in patients with portal hypertension and multiple hepatic lesions in highly selected cases. In addition, this algorithm also does not provide indications concerning second-line therapies, retreatment choices, or combined treatments. Furthermore, there are several differences in indication of Liver transplantation for HCC among countries. In Japan, it is considered that the therapeutic algorithm in the Japanese guidelines for the management of liver cancer is established and superior to the BCLC treatment algorithm in Japanese population [4]. HKLC from China needs further evaluations. Among these countries, treatment situations and options are various in some part, thus, it seems to be currently difficult to establish the unified staging system which provides both optimal treatment recommendation and prediction prognosis for worldwide.

Another goal of staging systems is to develop a globally applicable staging classification [87].

There is currently no globally accepted system for HCC, and thus no common language on which to base treatment decisions and guide research. For practical purposes, staging systems should be simple and based on data that are easily obtainable. Our novel $N \times S$ factor and $N \times S$ factor-based staging system are very simple and obtained anywhere and easily in daily practice, and it may potentially become one of a common score in many countries.

28.9 Conclusion

As mentioned above, many staging systems and scoring systems have been established and refined. However, there is currently no globally accepted system for assessing HCC patients, due to regional differences in tumor extension and underlying liver disease, which affects the patient prognosis, thus, a staging classification needs to be validated in both western and Asia-Pacific patient populations. Although the prognosis of HCC patients is complex for various reasons, simple staging systems available anywhere are needed at first to compare the differences of the prognosis of HCC patients among the nations.

In conclusion, further research efforts are needed for us to gain a full understanding of the factors that affect the prognosis of patients with HCC, and it will allow us to refine staging classifications and improve our therapeutic approach. Growing evidence of tumor biology and development in imaging techniques and treatment modalities against both HCC and liver disease will result in the proportion of better staging systems in the future.

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Part III
Therapies

Percutaneous Ethanol Injection

29

Tito Livraghi, Maria Franca Meloni, and Anita Andreano

Contents

29.1 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), radio frequency (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.

29.2 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 multi- hole 22 G needle and a phial of 95° ethanol (Fig. 29.1). Alcohol acts by two mechanisms. The first is due to its diffusion within the cells, which causes immediate dehydration of cytoplasmic proteins with consequent coagulation necrosis followed by fibrosis. The second is due to its

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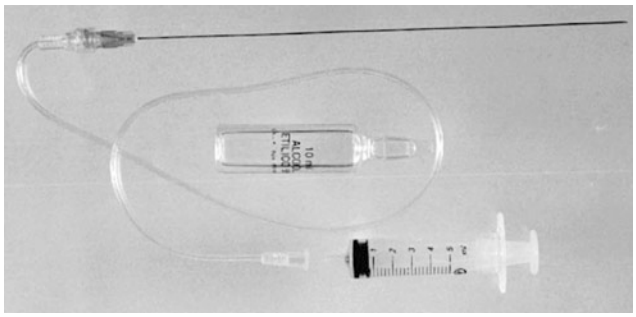

Fig. 29.1 Material used to perform PEI

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 presence of septa or even impossible in 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 lesion diameter of the lesion in centimeters [8]. The latter technique is adopted for more advanced HCC, single or multiple, that do 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 be also performed in selected patients with segmental or subsegmental portal thrombosis, injecting 1–3 ml of ethanol directly into to 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].

29.3 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 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) and spiral multi-slice CT (Fig. 29.2) with the triphasic technique (4–5 ml/s, 30, 70 and 120 s after the injection of contrast medium). We use other imaging techniques (angiography, MR, PET) or biopsy 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 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 shows 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 multi-session treatment as it permits to evaluate before each session if there is persistence of any viable area. The following instillation of ethanol can be therefore selectively performed in the tumoral tissue (Fig. 29.3).

As tumor markers, we use alpha-fetoprotein (AFP) and des-gamma-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 multi-session 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.

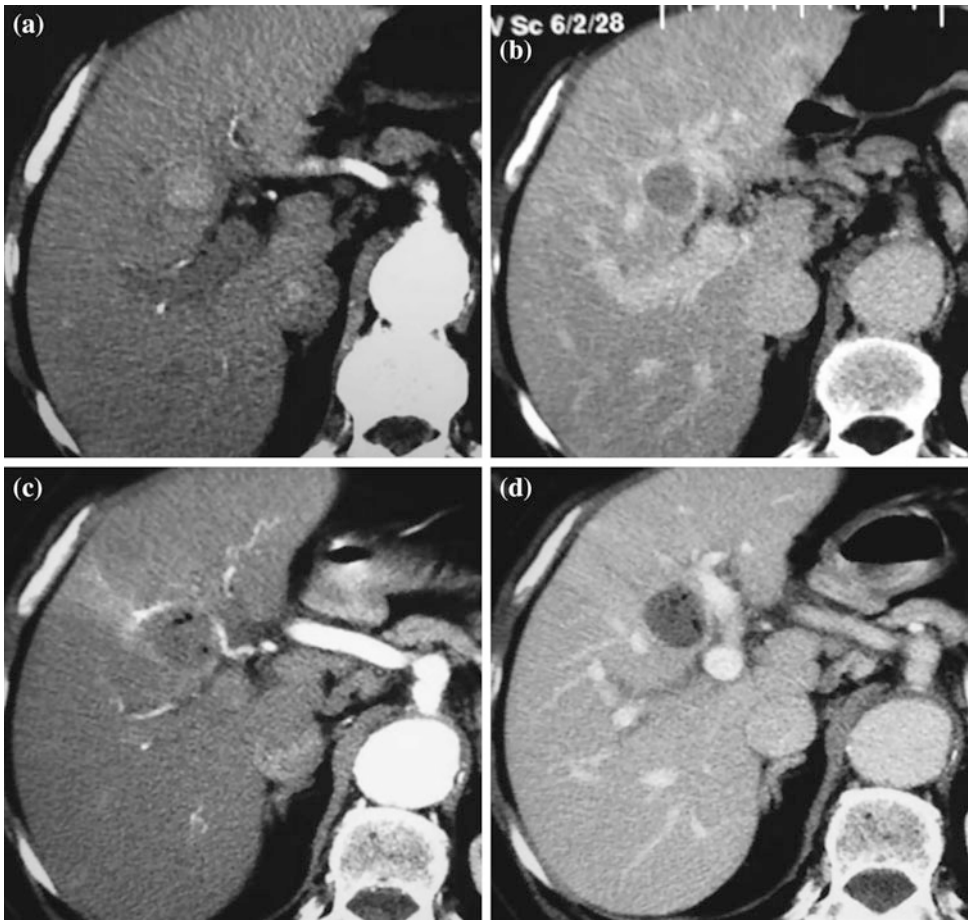

Fig. 29.2 Transverse CT scans showing a HCC of 2 cm in the right lobe treated with multi-session PEI. **a**, **b** Before treatment the tumor shows hypervascularity during the arterial phase and wash-out in the portal phase. **c**, **d** The arterial and portal phase CT scans the day after treatment show a completely necrotic lesion because of absence of enhancement. Very small bubbles of gas due to recent necrosis are detectable inside the treated area

29.4 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, 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, that 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 case 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.

29.5 Results

29.5.1 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]. Their 5-year survival, in patients with single HCC ≤ 5 cm or with ≤ 3 nodules ≤ 3 cm, ranged from 43 to 63 %. Recently, two papers from Japan reported 20-year outcome and prognostic factors related to 270 and 685 consecutive case series, respectively. Ebara, in patients with HCC with ≤ 3 nodules ≤ 3 cm, obtained an overall 3- and 5-year survival rates of 81.6 and 60.3 %, respectively, with 0 % of treatment mortality and 2.2 % of major complications. The rates were

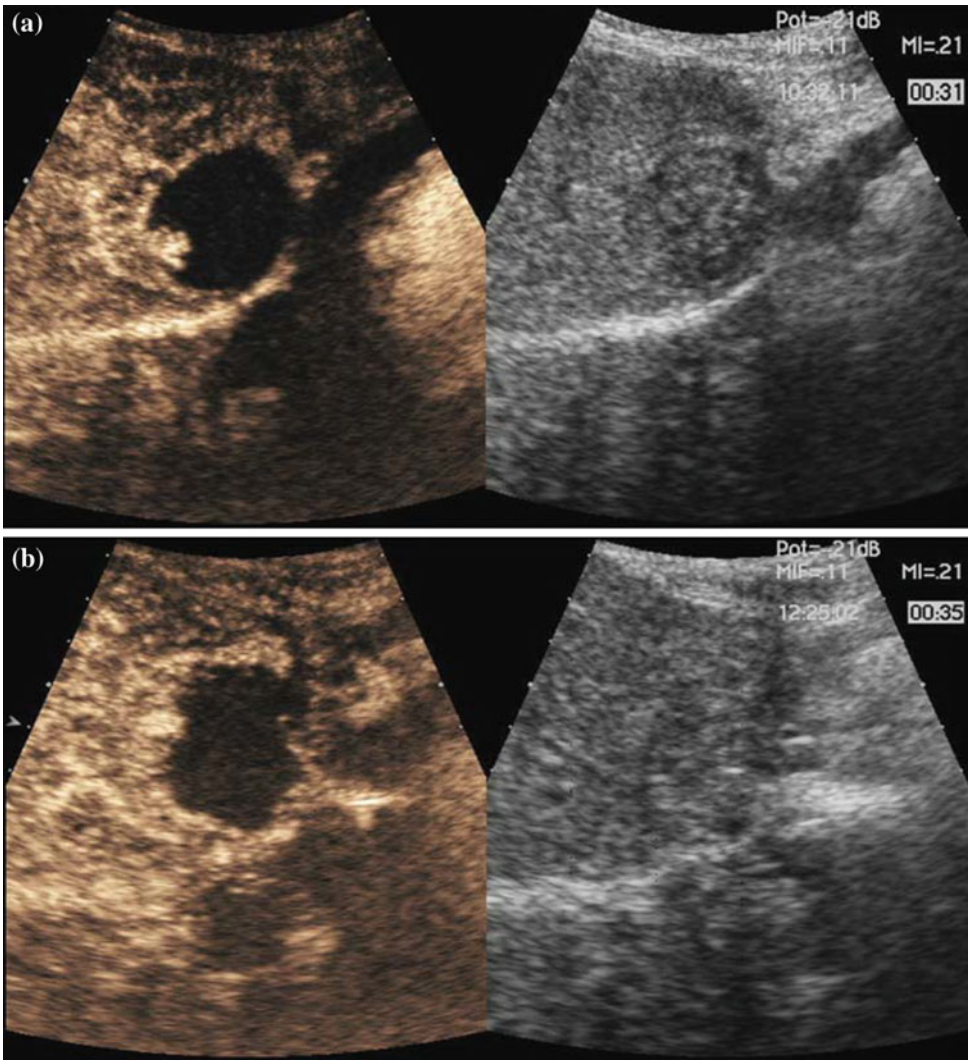

Fig. 29.3 Contrast-enhanced US scans showing a HCC nodule treated with multi-session PEI. **a** Vital hypervascularized tissue remains present after the first session. **b** After the second treatment, targeted using contrast enhanced US as guidance, the lesion is completely treated

higher, i.e., 87.3 % at 3 and 78.3 % at 5-year, in Child A patients with a solitary tumor ≤ 2 cm in diameter [17]. Shiina obtained a complete ablation in 98.5 % of cases (99 % in tumors <2 cm), while the treatment mortality was 0.06 %. In patients with HCC with ≤ 3 nodules ≤ 3 cm the overall survival was similar, i.e., 59.5 % [19].

In all the studies main pretreatment factors influencing survival resulted liver function, tumoral markers (AFP, DCP) level, number and size of tumors. A post-treatment prognostic factor is the complete response to PAT [20]. The main cause of death in Child's A patients was progression of neoplastic disease due mainly to the appearance of new lesions, while in Child's 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 %.

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 [21].

29.5.2 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 [22, 23]. 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 [24–26]. Another RCT on 184 patients with HCC ≤ 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 statistical significant difference was reported between PEI and PAI [27].

For explaining the difference regarding these parameters, it is important to remember that also at the earliest stages, 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) [28–30]. 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 [29–32]. Better long-term results of RF are due to the fact that termoablation 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. However, a recent study selecting 254 patients with single HCC ≤ 2 cm for propensity score matching analysis, demonstrated that PEI and RF were substantially effective in terms of 5-year survival, 64.7 and 72.9 %, respectively, despite higher cumulative and local recurrence rates of PEI [33]. As expected, RF resulted superior to PEI also in tumor of medium and large size [34].

Some retrospective studies comparing PEI and hepatic resection (HR) showed 5-year survival rates broadly equivalent, with an approximate rate of 50 % for both [35–38]. These data were confirmed by the only RCT which compared patients with one or 2 nodules ≤ 3 cm in size, which did not find any statistical difference for recurrence rate and survival [39].

29.5.3 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 [40, 41]. The combination of repeated single-session PEI and trans-arterial-chemo-embolization (TACE) has been compared to repeated single-session PEI in patients with unresectable HCC [42]. The combination of TACE and PEI was associated with a longer survival (1, 3, 5-year survival: 90, 52, and 43 %) compared to PEI treatment alone (1, 3, and 5-year survival: 65, 50, and 37 %). Validity of this combination was recently confirmed by a meta-analysis of ten RCTs including 595 patients with unresectable HCC. The pooled result showed that TACE plus PEI compared with that of TACE alone improved the 3-year overall survival [43].

29.6 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 multi-centricity is already present in 50 % of early stages and that 93 % of patients with single minute HCC presented other nodules within 5 years [44]. Being multi-centric 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 was re-resected [44].

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 [36–38] and the recent RCTs comparing HR and PATs demonstrated roughly equivalent results [39, 46, 47]. 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 around 50 % after HR [48]. 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 [49, 50], 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 centre follows a personal algorithm for such borderline patients. Currently, RF is becoming the gold standard for nodules <2 cm [51], 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

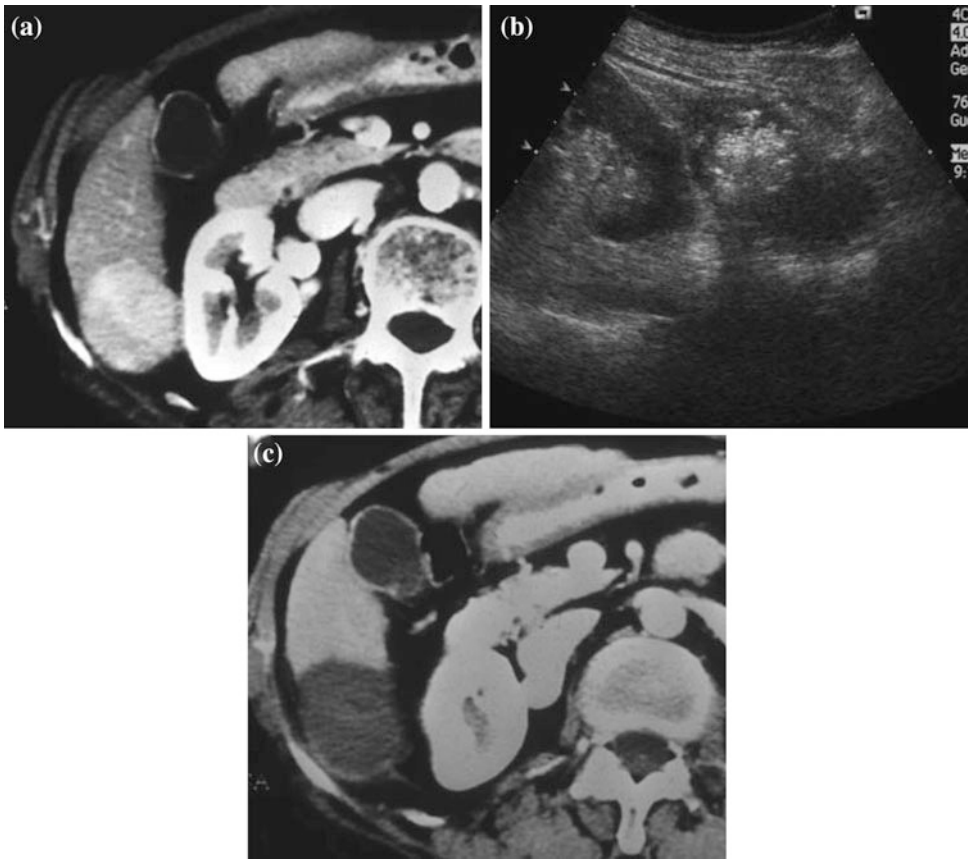

Fig. 29.4 Transverse CT and US scans showing a HCC of 4.2 cm located in segment VI, close to the bowel, treated with single-session PEI because of its at risk location. **a** At the baseline the lesion appears well vascularized at arterial phase CT scan. **b** US scan at the end of the procedure shows the hyperechoic zone of ethanol filling the tumor. **c** At the arterial phase CT scan one month after treatment no enhancement is visible within the tumor

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, 52].

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. 29.4) (above all when fibrotic adhesions between the hepatic capsule and intestinal wall are suspected, because of the risk of perforation). Combined therapies have been also proposed for these kinds of lesions [53, 54]. PEI is also useful to treat lesions closed to large vessels, as it is not affected by the so-called sink effect. PEI remains a good indication to treat segmental portal thrombosis [10]. PEI is also used for downstaging patients with an advanced HCC exceeding the UCSF/Milan criteria for liver transplantation [55]. In our experience, we downstaged a patient with alcoholic cirrhosis Child B class, exceeding the Milan criteria because carrier of eight nodular HCC <2 cm in size. Under general anesthesia, six of the nodules were treated with RF and two with PEI because at risk for RF. Because CT examination showed a complete response the patient was transplanted, and the histological examination confirmed the complete ablation.

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 [56]. 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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Thermal Ablative Treatments for Hepatocellular Carcinoma

30

Antonio Facciorusso and Michele Barone

Contents

30.1 Introduction

Hepatocellular carcinoma (HCC) represents the third most common cause of cancer-related death and the leading cause of mortality among patients with cirrhosis [1, 2]. Thanks to recent improvements in surveillance protocols and diagnostic tools, early HCC diagnosis is currently feasible in 30–60 % of cases [3].

Local ablation is considered the first-line treatment option for patients at early stage, who are not amenable to surgery or orthotopic liver transplantation (OLT). Among ablative treatments, thermal ablative therapies have gained an increasing role in the last decade due to their efficacy in preventing local recurrence as well as in prolonging overall survival (OS). Thermal ablative treatments are classified as hyperthermic (heating of tissue at 60–100 °C), such as radiofrequency ablation (RFA), microwave ablation (MWA), high-intensity focused ultrasound (HIFU) or laser therapy, or hypothermic (freezing of tissue at −20 °C up to −60 °C), such as cryoablation.

These procedures are usually performed by means of a percutaneous approach but in particular conditions (for instance in cases of nodules in "at-risk" location) laparoscopic ablation may be recommended.

In this chapter, we aim to provide a comprehensive overview on the main thermal therapies for HCC with the up-to-date data on their efficacy and safety.

30.2 Indication to Treatment

Thermal ablative treatments represent the standard of care for unresectable HCC in very early/early stage according to Barcelona Clinic Liver Cancer (BCLC) system [2, 4]. The term "unresectable" covers a broad spectrum of pathological conditions, from single nodule in a deep location (therefore not easy to treat by surgery) to multinodular disease in patients with deteriorated liver function. Therefore, percutaneous therapies are a valuable option in nonoptimal

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Table 30.1 Contraindications for thermal ablative treatments

Absolute contraindications
1. Extrahepatic disease
2. Altered mental status
3. Active infection
4. Tumor abutting a major hepatic duct
5. Liver decompensation (particularly in presence of ascites)
Relative contraindications
1. Lesions >5 cm
2. More than four lesions
3. Severe pulmonary or cardiac disease
4. Refractory coagulopathy

candidates to surgery due to tumor size, number, location, liver function, or comorbidities.

Another indication to thermal treatment is the pretransplant setting, where RFA has been proved to be effective both as downstaging and bridging therapy [5–7].

Main absolute and relative contraindications to thermal treatments are described in Table 30.1. Absolute contraindications, shared with other locoregional treatments, are the presence of extrahepatic liver disease, altered mental status, active infection, tumor abutting a major hepatic duct, impaired liver function (particularly in presence of ascites); relative contraindications are more than four nodules or at least one lesion >5 cm, severe cardiopulmonary disease, and refractory coagulopathy [8].

30.3 Mechanism of Action and Equipment for Radiofrequency Ablation

RFA has been the most widely adopted thermal therapy because of its well-proven effectiveness and relative safety, with a 5-year survival rate of 40–70 % in early stage HCC patients [9, 10].

However, the best outcomes have been reported in HCCs classified as BCLC stage 0 (i.e., single nodule ≤ 2 cm) for which RFA has demonstrated a competitive efficacy even with respect to surgery in terms of OS (see below) [11, 12].

The mechanism of action of RFA relies on the destruction of tumoral tissue by the radiofrequency-generated heat. In particular, the injury is due to frictional heat produced by the ionic agitation of particles within tissue as a consequence of the application of alternating current [13].

The electrical alternating current in the radiofrequency range (200–1200 MHz) is delivered by a needle electrode under imaging guidance (usually ultrasonography) and the electrical circuit is completed through grounding pads attached to the thighs or back of the patient. The needle is partially insulated and presents an activated tip that is not insulated. This tip varies in length with the most common size being 3 cm long. Tips may be singular and straight or consisting of an array of expandable tines that form an umbrella fully encompassing the nodule when deployed.

Table 30.2 describes main advantages of each device, although definitive evidence of a superiority of one device over the others in determining coagulation necrosis is still lacking [14]. Unlike the previously mentioned LeVeen electrodes, cooled needle is internally perfused by chilled water during ablation, cooling adjacent tissue, and preventing charring around the electrode tip [15, 16]. In fact, tissue boiling and charring around the electrode acts as electrical insulator and limits the ablation area through increased impedance. To overcome this inherent limitation, several devices, described in Table 30.2, have been developed, each with its own advantages and disadvantages.

An important aim of the treatment should be to ensure thermal destruction not only of the tumoral nodule but also of a surrounding margin about 1 cm long in order to ablate eventual microsatellites, thus preventing local recurrence.

In order to reach this target, multiple overlapping ablations or simultaneous application of multiple electrodes can further enlarge the ablation zone, thus allowing ablation of nodules up to 4–5 cm.

Another aspect to be considered is the "heat sink effect," namely the dissipation of the thermal output by blood flowing through adjacent vessels decreasing the efficacy of the procedure [17]. This is the reason why nodules close to major vessel are considered a suboptimal target and constitute a relative contraindication for RFA.

The procedure is usually performed under sedation when the percutaneous approach is preferred. In cases of laparoscopic RFA, to be considered in cases of nodules close to the liver capsule or other organs, general anesthesia is needed.

At our Center, we usually perform RFA with a 150 W generator connected to an expandable 15–14-gauge

Table 30.2 Equipments used in radiofrequency ablation

Company	System name	Electrode	Mechanism	Advantages
AngioDynamics	StarBust XL®	Deployable tines	Expands once inside the tumor	Larger area of necrosis
RadioTherapeutics	RF Ablation System®	Deployable tines	Expands once inside the tumor	Larger area of necrosis
Radionics	Cool-tip RF System®	Straight needle	No expansion	Minimal tissue charring
Berchtold	Elektrotom 106 HFTT®	Straight needle	No expansion	Minimal tissue charring

electrode with a 2.0-cm-long exposed tip (expandable by means of seven hooks). After administration of analgesia (50–60 mg of propofol and 0.05–0.1 mg of fentanyl) as well as local anesthesia (5–15 mL of 1 % lidocaine) by an anesthesiologist, we insert RFA needle into the tumor placing the electrode into the center of the lesion and maintaining the temperature of the needle tip at 80–100 °C for 10–12 min. After ablation, the needle is retracted maintaining its tip hot in order to prevent, by thermal coagulation, seeding, or hemorrhage along the electrode track. For medium and large nodules, different applicator positions are usually adopted to create overlapping coagulation zones. No antibiotic prophylaxis or anti-inflammatory drugs are administered prior to therapy [18].

For follow-up, it is our practice to obtain CT scan at 2 and 6 months after the procedure in addition to alpha-fetoprotein (AFP) measurement in case of elevated pretreatment values of this marker and liver function tests assessment.

30.4 Survival Outcomes After RFA for HCC

A large number of studies have confirmed the efficacy of RFA in early HCC patients suggesting this procedure as viable therapeutic option in unresectable early stage. Considering the state of art of the literature, RFA provided 5-year survival rates of 40–70 % and beyond in HCC series [9, 10].

A recent Chinese study reported overall survival rates of 96.6, 60.2, and 27.3 % at 1, 5, and 10 years [19], similar to those reported by Kim et al. [20] which were 95.5, 59.7, and 32.3 %, respectively. These results are concordant with other recent Western studies conducted in patients within Milan criteria (87.0–99.0 % at 1 year, 60.0–87.4 % at 3 years, and 42.3–74.8 % at 5 years) [21, 22].

Several studies pointed out different predictors of survival, such as Child-Pugh (CP) score, initial response, serum ferritin, number or size of nodules, and AFP levels [22–24].

Our group has recently analyzed predictors of post-recurrence survival (PRS) after RFA, namely the survival time elapsed after tumor recurrence [21]. We found, in line with other studies, baseline CP score, AFP levels, and Performance Status (PS) as predictors of OS in multivariate analysis. However, analysis of PRS showed that in addition to CP score and PS, also tumor burden at the time of recurrence and recurrence pattern had a significant influence on PRS [21]. On the other hand, AFP level, a major prognostic variable for OS at baseline, became not significant when assessed at recurrence, demonstrating that factors affecting OS evaluated at baseline are different from those at tumor relapse [21].

It is noteworthy that the occurrence of local recurrence (LR) had no significant influence on survival in our study [21] as well as in other reports [20, 24, 25], probably due to the frequent multifocality of distant recurrences that makes more difficult the therapeutic approach, while local recurrences, even when multifocal, are confined in one liver segment (namely the same as that previously treated) and may be more easily treated with RFA or a single selective transarterial chemoembolization (TACE) session.

Unlike OS, reported rates of LR after RFA are not univocal ranging from 3.2 to 27 % at 5 years [19–24], maybe because of different etiologies of HCC in the published series, different strategies for coping with an insufficient ablative margin, use of combined treatment with TACE and, above all, different definition of radiologic tumor recurrence at imaging. As expected, tumor features such as nodules number, size, histopathological grading, and AFP have been found to be predictors of recurrence [19–24]. Moreover, an insufficient ablation margin after the treatment appears to be an important prognostic factor for LR [26, 27].

Intrahepatic distant recurrences occur very frequently, from 68 to 74 % at 5 years [19–22, 24], and are usually associated to poorer prognosis. This type of recurrence is predominantly related to underlying hepatic disease and is often observed after 2 years, which is the time point considered able to differentiate between real recurrences from de novo tumors occurred in the pro-tumorigenic milieu of liver cirrhosis [28].

Therefore, because of their high frequency and aggressive behavior, distal recurrences are a major determinant of patient survival.

30.5 Prevention of Recurrence After RFA

In the last years there has been an increasing interest in adjuvant drugs able to decrease the high rates of tumor recurrence observed after RFA.

Despite the promising results of some pilot reports [29, 30] and the theoretical advantages of sorafenib (Nexavar®, Bayer, Leverkusen, Germany) in adjuvant setting, a broad multicenter randomized controlled trial [Sorafenib as Adjuvant Treatment in the Prevention Of Recurrence of Hepatocellular Carcinoma (STORM)], enrolling 1114 HCC patients after resection or RFA, failed to find a significant improvement in recurrence-free survival [Hazard Ratio (HR): 0.940, 95 % Confidence Interval (CI): 0.78–1.13, $p = 0.26$] and OS (HR 0.99, 95 %CI 0.76–1.30, $p = 0.48$) [31]. This disappointing result was partly related to the high discontinuation rate of therapy (24 % vs. 7 % of placebo) and consent withdrawal (17 % vs. 6 %) in the sorafenib arm, mainly because of severe adverse events [31].

Likewise, other drugs, such as interferon, provided discordant results in the adjuvant setting due to their high cost and narrow therapeutic window [32, 33].

Therefore, most of the recent research in this field has focused on other drugs. On the basis of the well-described pro-tumorigenic and profibrogenic properties of angiotensin II, due to the induction of vascular endothelial growth factor (VEGF) and transforming growth factor-beta 1 (TGF-β1) release [34, 35], broad clinical reports have found significantly decreased HCC recurrence rates after RFA when angiotensin converting enzyme inhibitor (ACE I) were used in combination with other agents such as branched-chain amino acids or vitamin K [36–38]. However, ACE I was effective in monotherapy and, above all, no significant difference in overall survival was observed in comparison to the control arm [36–38].

Our group has recently published a retrospective report conducted in 153 HCC patients treated with RFA, finding a significant benefit both in terms of recurrence and OS in hypertensive subjects in treatment with angiotensin II type 1 receptor blockers (sartans) as compared to those under ACE I therapy and to non-hypertensive subjects [39]. The apparent superiority of sartans over ACE I may be due to the selective inhibition of angiotensin II receptor 1, responsible of the profibrogenic and proangiogenic activity of angiotensin, while proapoptotic and antitumorigenic activity of receptor 2 is preserved and even enhanced in patients administered sartans unlike ACE I which prevents the binding of angiotensin II to both receptors [40]. However, these preliminary results still need further confirmation.

In conclusion, in spite of the great amount of published reports and in absence of broad RCTs, clear evidence in favor of an adjuvant treatment after RFA is still lacking.

30.6 Adverse Events of RFA

In a recent systematic review of 9531 patients treated with RFA, treatment-related severe adverse events were registered in 4.1 % of cases with a mortality rate of 0.15 % [41].

Adverse events include gastrointestinal tract injury with/without perforation (0.06–0.3 %), diaphragm injury (0.03 %), pleural effusion (0.2–2.3 %), bile duct stricture (0.06–0.5 %), biloma (0.06–0.96 %), gallbladder injury (0.0–0.1 %), and hepatic infarction (0.03–0.06 %) caused by vascular injury or thrombosis. Other complications, related to direct mechanical injury, are tumor seeding (0.27 %), tumor rupture (0.3 %), hemoperitoneum (0.3–1.6 %), and hemo/pneumothorax (0.15–0.8 %). Events not related to mechanical or thermal injury to the liver are hepatic abscess (0.1 %), grounding pad burn (0.6 %), and vasovagal reflex (0.1 %) [42]. However, considering the low incidence of all these complications, RFA can be considered a safe procedure in high-volume centers when proper indications to treatment are followed.

30.7 RFA in Pretransplant Setting

In the recent years, RFA has gained increasing interest either as bridging or as downstaging therapy prior to transplantation in HCC patients. A number of studies have reported complete tumor necrosis rates at pathological evaluation of the explanted liver up to 47–75 % [5–7, 43, 44].

In particular, complete necrosis rate ranges between 50 and 78 % in nodules within 3 cm and between 13 and 43 % in larger neoplasms [5–7, 43, 44] versus 27–57 % of TACE in Milan-in patients [45, 46].

Safety concerns previously raised by some authors due to the theoretical risk of tumoral seeding, reported to occur in about 3 % of cases [47], have been recently overcome [48]. Therefore, although TACE remains the most used treatment before OLT, RFA has to be preferred in cases of single nodules under 3 cm as provides higher complete necrosis rates and lower risk of recurrence after transplantation [49].

30.8 RFA Versus Liver Resection for HCC

Surgical resection is the first-line option in very early/early patients who do not fulfill transplant criteria [2–4]. However, no more than 10–35 % of patients are actually suitable to surgery due to tumoral burden, inadequate hepatic reserve, or overall poor clinical status [2, 4]. These patients may be offered RFA as viable option because of its proven efficacy.

The aforementioned excellent clinical outcomes of RFA have recently opened debates on whether RFA can replace resection as a first-line therapy, particularly in very early patients (namely, those with a single nodule less than 2 cm). To answer this question, many investigators have performed cohort studies or randomized controlled trials (RCTs) that directly compared the two methods.

Table 30.3 reports the main characteristics of the three RCTs comparing the two treatments published so far. In the RCT by Chen et al., enrolling 71 patients submitted to RFA and 90 submitted to resection with single HCC <5 cm, no difference was observed between the two groups either according to 3-year overall survival (71.4 % vs. 73.4 %) and to disease-free survival (DFS) (64.1 and 69.0 %) [50]. Authors stated that these findings were confirmed even when stratifying by tumor size but did not provide survival outcomes nor p values [50].

Huang et al. conducted a RCT in 230 Milan-in HCC patients reporting significantly lower 5-year overall survival rate (54.8 % vs. 75.7 %, $p = 0.001$) and recurrence-free survival rate (28.7 % vs. 51.3 %, $p = 0.017$) after RFA with

Table 30.3 Randomized controlled trials comparing radiofrequency ablation and surgery in hepatocellular carcinoma patients

Study	Liver function	Tumor features	Treatment	3-year SR (%)	5-year SR (%)	3-year DFS (%)	5-year DFS (%)
Chen [50]	CP A ICG-R15 < 30 % PLT > 40,000/mm^3	Single < 5 cm	HR 90 RFA 71	73.4 71.4	NA NA	69 64.1	NA NA
Huang [51]	CP A/B ICG-R15 < 20 % PLT > 50,000/mm^3	Within MC	HR 115 RFA 115	92.2 69.6	75.7 54.8	60.9 46.1	51.3 28.7
		Single $\leq$3 cm	HR 45 RFA 57	95.6 77.2	82.2 61.4	NA NA	NA NA
		Single 3–5 cm	HR 44 RFA 27	95.5 66.7	72.3 51.5	NA NA	NA NA
		Multifocal <3 cm	HR 26 RFA 31	80.8 58.1	69.2 45.2	NA NA	NA NA
Feng [52]	CP A/B ICG-R15 < 30 % PLT > 50,000 mm^3	Up to 2 nodules <4 cm	HR 84 RFA 84	74.8 67.2	NA NA	61.1 49.6	NA NA

Abbreviations: *SR* survival rate; *DFS* disease-free survival; *CP* child-pugh; *ICG-R15* indocyanin green retention at 15 min; *PLT* platelets; *HR* hepatic resection; *RFA* radiofrequency ablation; *NA* not available; *MC* milan criteria

respect to surgery [51]. The benefit of resection was maintained when patients were stratified by tumor size and number (Table 30.3).

On the other hand, the trial by Feng et al., enrolling 168 patients with up to 2 nodules less than 4 cm, showed a 3-year overall survival rate of 67.2 % after ablation and 74.8 % after surgery ($p = 0.342$), whereas the corresponding 3-year recurrence-free survival rates were 49.6 and 61.1 %, respectively ($p = 0.122$) [52]. No stratification for tumor stage was provided in this study.

Thus, the available RCTs report discordant results with the sole study by Huang et al. demonstrating a superiority of hepatic resection over RFA [51]. However, the different proportions of nodules beyond the very early stage are likely to be responsible of these conflicting results, since it is known that ablation beyond this stage is less able to achieve complete tumor necrosis.

None of the aforementioned RCTs restricted their analysis to single nodules $\leq$2 cm, while there are five observational studies on this specific setting [53–57]. Unfortunately, most of these retrospective studies suffer from selection bias as RFA patients tended to be older and to present more deteriorated liver function than surgical ones, while larger nodules were more likely to be treated with resection. Thus, results in terms of both patient survival and recurrence rate can be biased by covariate distribution. Two of these studies, which tried to obviate to such a bias by means of propensity score one-to-one match, reported better DFS in surgical patients ($p = 0.031$ and $p < 0.001$) but discordant results with regard to overall survival ($p = 0.296$ and $p = 0.034$, respectively) [54, 57]. However, several concerns have been raised on the rigorousness of the statistical procedure adopted, hence such findings require further confirmation [58]. The low level of evidence impairs the findings of several meta-analyses published in this field, which mostly support the superiority of hepatic resection over RFA in early stage without significant differences in single nodules less than 2 cm [59, 60].

An interesting study conducted by the Bologna group, based on a Markov model and a Monte Carlo probabilistic sensitivity analysis, demonstrated that in a 10-year perspective RFA provided similar life expectancy and quality-adjusted life expectancy at a lower cost than resection in very early HCC patients, hence it was the most cost-effective therapeutic strategy for this stage [61]. In the presence of two or three nodules $\leq$3 cm, life expectancy and quality-adjusted life expectancy were very similar between the two treatments, but cost-effectiveness was again in favor of RFA [61]. Therefore, the authors concluded that RFA is more cost-effective than resection for very early HCC and in the presence of two or three nodules $\leq$3 cm, while surgical resection remains the best strategy for single larger early stage HCCs [61].

In conclusion, as supported by a decision-making analysis performed by the same group, the superiority or equivalence of a treatment over the other is strictly dependent on the nonlinear relationship among tumor number, size, and liver function, with RFA to be preferred in cases of smaller tumors and impaired liver function [62].

30.9 RFA Versus Percutaneous Ethanol Injection (PEI) in Early HCC Patients

PEI is a well-established technique for the treatment of small HCCs and induces coagulative necrosis as a result of cellular dehydration and protein denaturation. However, ethanol

Table 30.4 Randomized controlled trials comparing radiofrequency ablation and percutaneous ethanol injection in hepatocellular carcinoma patients

Study	Region	Patients *n*	Nodules *n*(1/> 1)	Tumor size, cm	Number of sessions	Complete response (%)	3-year survival (%)	3-year recurrence (%)
Lin [67]	Taiwan	RFA (52) PEI (52)	38/14 40/12	2.9 ± 0.8 2.8 ± 0.8	1.6 ± 0.4 6.5 ± 1.6	96.0 88.0	74 50	18 45
Lin [68]	Taiwan	RFA (62) PEI (62)	49/13 49/13	2.5 ± 1.0 2.3 ± 0.8	1.3 ± 0.3 4.9 ± 1.3	96.1 88.1	74 51	14 34
Shiina [69]	Japan	RFA (118) PEI (114)	72/46 60/54	NA NA	2.1 ± 1.3 6.4 ± 2.6	100.0 100.0	81 66	1.7 11
Wang [70]	China	RFA (49) PEI (49)	NA NA	2.4 ± 1.2 2.3 ± 1.4	NA NA	93.8 77.5	NA NA	NA NA
Azab [71]	Egypt	RFA (30) PEI (30)	NA NA	NA NA	1.45 7.68	85.0 75.0	NA NA	NA NA
Giorgio [72]	Italy	RFA (128) PEI (143)	128/0 143/0	2.3 ± 0.4 2.2 ± 0.5	5 8	100.0 100.0	83 78	7.8 9.4
Lencioni [73]	Italy	RFA (52) PEI (50)	40/12 31/19	2.8 ± 0.6 2.8 ± 0.8	1.1 ± 0.5 5.4 ± 1.6	91.0 82.0	NA NA	21 59
Brunello [74]	Italy	RFA (70) PEI (69)	54/16 54/15	2.4 ± 0.5 2.2 ± 0.5	NA NA	95.7 65.6	59 56	NA NA

Abbreviations: *RFA* radiofrequency ablation; *PEI* percutaneous ethanol injection; *NA* not available

diffusion is likely to be impaired by intratumoral fibrotic septa in cases of nodules larger than 2 cm.

In fact, the efficacy of such a technique in early stage (namely, multiple nodules or single nodule larger than 2 cm) is considerably inferior as compared to RFA with a complete necrosis rate of 70 % in nodules of 2–3 cm and 50 % in those between 3 and 5 cm [63, 64]. On the other hand, RFA showed a significantly higher necrosis rate, up to 71 % in noninfiltrating medium-size (i.e., between 3 and 5 cm) nodules [65]. In our recently published experience, overall complete necrosis rate after RFA was 84.4 % in a series whose median tumor size was 3 cm [21, 23].

However, even if it is widely recognized the superiority of RFA over PEI in medium-size and large nodules, a clear advantage in term of survival in small HCCs (less than 3 cm) is still unclear.

In fact, a recent meta-analysis including 8 RCTs, found better survival outcomes (HR: 0.67, 95 %CI: 0.51–0.87, $p < 0.001$) and a lower 3-year LR rate [Risk ratio (RR) 0.41, 0.30–0.57, $p < 0.01$] after RFA as compared to PEI [66]. However, but sensitivity analysis confirmed the superiority of RFA only in Asian studies [67–71] while the three included Italian studies [72–74] found only a nonsignificant trend in favor of RFA as for survival (HR 0.82, 95 %CI 0.56–1.20, $p = 0.30$) [66]. Table 30.4 summarizes the main findings of the aforementioned trials. Quite interestingly, RFA provided similar if not better results as compared to PEI requiring a significant lower number of sessions (Table 30.4). This aspect has to be taken into account since, although a single PEI treatment has significantly lower costs than RFA, the higher number of PEI sessions reduces this benefit and increases the risk of tumoral seeding.

The above-described results are in agreement with another systematic review including four RCTs comparing the two techniques in small HCCs under 3 cm which, however, found RFA associated to higher major complication rates and to be more costly than PEI [75].

In conclusion, although the fact that RFA leads to better survival rates than PEI in small HCCs is still matter of debate, the lower local recurrence rate stands for a wider application of RFA in hepato-oncology.

30.10 Combined Treatment

There is increasing evidence that combining RFA to TACE may increase the therapeutic benefit in larger HCCs. In fact, the two techniques may exert a synergistic effect on inducing nodule necrosis: occlusion of the tumor arterial supply by TACE would increase the area of coagulation necrosis obtained by RFA minimizing heat loss whereas the heating-related reactive hyperemia induced by RFA would concentrate the chemotherapeutic agent released during TACE in the peripheral residual viable neoplastic tissue and would reduce cell resistance to the drug [76].

A recent meta-analysis of eight RCTs [77–84] including 598 patients indicated that RFA plus TACE determines a significantly higher 3-year overall survival rate [Odds Ratio (OR): 2.65, 95 %CI: 1.81–3.86, $p < 0.001$] and 3-year RFS rate (OR: 3.00, 95 %CI: 1.75–5.13, $p < 0.001$) than RFA

Table 30.5 Randomized controlled trials comparing transarterial chemoembolization combined to radiofrequency ablation versus radiofrequency ablation alone in hepatocellular carcinoma patients

Study	Region	Patients *n*	Tumor size, cm	CP A/B/C	3-year survival (%)	3-year recurrence (%)
Peng [77]	China	TACE + RFA (69) RFA (70)	≤5.01 –	60/9/0 59/11/0	69 47	45 18
Cheng [78]	China	TACE + RFA (96) RFA (100)	≤7.5 –	NA NA	55 32	NA NA
Yang [79]	China	TACE + RFA (24) RFA (12)	6.6 ± 0.6 5.2 ± 0.4	NA NA	NA NA	NA NA
Shibata [80]	Japan	TACE + RFA (16) RFA (13)	1.7 ± 0.6 1.6 ± 0.5	32/14/0 33/10/0	84.8 84.5	48.8 29.7
Morimoto [81]	Japan	TACE + RFA (19) RFA (18)	3.6 ± 0.7 3.7 ± 0.6	12/7/0 16/2/0	93 80	NA 28
Kang [82]	China	TACE + RFA (19) RFA (18)	6.7 ± 1.1 6.2 ± 1.2	12/7/0 12/6/0	36.8 16.7	NA NA
Shen [83]	China	TACE + RFA (18) RFA (16)	5.6 (2.2–15.8) 5 (2.3–12.3)	4/14/0 6/10/0	73.3 20.4	50 18.7
Zhang [84]	China	TACE + RFA (15) RFA (15)	4.6 (2.3–7.1) 4.1 (2.4–6)	NA NA	NA NA	NA NA

Abbreviations: *CP* child-pugh; *TACE* transarterial chemoembolization; *RFA* radiofrequency ablation; *NA* not available

alone, with no difference in major complications (OR: 1.20, 95 %CI: 0.31–4.62, $P = 0.79$) [85]. Subgroups analysis revealed that most of this benefit was obtained in patients with intermediate- and large-size HCCs, which are likely to be the optimal setting for the combined treatment [85]. These results should be considered with caution as all the included studies had been conducted in Asia with conventional TACE (see Table 30.5), hence the applicability of such findings in the West is still unclear, although a recent small Italian retrospective report confirmed the superiority of RFA combined to drug-eluting beads TACE over RFA alone in single HCCs beyond 3 cm [86].

30.11 Other Thermal Ablation Techniques

30.11.1 Microwave Ablation

Microwave ablation (MWA) aims to induce tumor necrosis using high frequency (>900 MHz, usually 2450 MHz) electromagnetic energy which determines continuous rotation of dipole molecules in the microwave's oscillating electric field. This vigorous movement of dipoles (mainly water molecules) generates friction and heat, thus inducing tissue death via coagulation necrosis [87].

In comparison to RFA, MWA has several theoretical advantages: it induces a broader zone of active heating, leading to higher temperatures within the targeted area in a shorter treatment time as it is not impaired by tissue desiccation and charring [88]; it is less affected by heat sink effect, because the cooling effect of blood flow is more pronounced within the zone of conductive rather than active heating [89]; multiple antennae can be simultaneously activated without the electrical interference phenomena observed in RFA, thus allowing more rapid treatment of large or multifocal tumors [89]. On these premises, MWA mostly shares the applications of RFA, with the above-cited advantages in larger nodules and/or close to blood vessel.

Three cohort studies demonstrated a complete ablation rate of 89–94 % and a 5-year survival rate of 51–57 % in predominantly Child-Pugh class B cirrhosis [90–92].

The safety concerns raised on the risks of the procedure, due to the broader and less predictable necrosis areas induced by MWA, have been recently overcome by a large multicenter Italian study conducted in a series of 736 patients, of which 522 with HCC, where MWA determined a major complication rate of 2.9 % with a periprocedural mortality rate of <0.01 % [93].

There are actually seven studies (of which one RCT) directly comparing MWA and RFA in HCC patients (94–100, Table 30.6). Unfortunately, the sole RCT published did not report long-term survival data but only complete necrosis rates, which were similar in the two treatment groups (89 % for MWA vs. 96 % for RFA) [94]. Retrospective studies reported heterogeneous results, particularly with regard to local recurrence probably because of different follow-up time length or radiologic criteria adopted (Table 30.6).

The sole meta-analysis published so far in this field reported no difference in local recurrence rates between RFA and MWA (OR: 1.01, 95 %CI 0.67–1.50, $p = 0.9$), as well as in complete ablation, 3-year overall survival and major adverse events ($p > 0.05$ for all) [101]. In subgroup analysis,

Table 30.6 Studies comparing radiofrequency ablation and microwave ablation in hepatocellular carcinoma patients

Study	Arm (*N*)	Study design	Region	CP (A/B/C)	Tumor size (cm)	Number nodules	3-year survival (%)	Local tumor recurrence (%)
Shibata [94]	RFA (36) MWA [36]	RCT	Japan	21/15/0 19/17/0	1.6 (0.7–2) 1.7 (0.8–2)	1.08 1.14	NA NA	8.3 17.4
Lu [95]	RFA (53) MWA (49)	R	China	49/4/0 39/10/0	2.6 (1–6.1) 2.5 (0.9–7.2)	1.35 2	37.6 50.5	20.9 11.8
Ohmoto [96]	RFA (34) MWA (49)	R	Japan	20/11/3 31/14/4	1.6 (0.7–2) 1.7 (0.8–2)	1.08 1.14	49 70	9 19
Ding [97]	RFA (85) MWA (113)	R	China	49/36/0 75/38/0	2.38 (1–4.8) 2.55 (0.8–5)	1.15 1.15	77.6 82.7	5.2 10.9
Zhang [98]	RFA (78) MWA (77)	R	China	78/0/0 77/0/0	NA NA	1.24 1.36	64.1 51.7	11.8 10.5
Abdelaziz [99]	RFA (45) MWA (66)	R	Egypt	24/21/0 25/41/0	$2.95 \pm 1.03^{+}$ 2.9 ± 0.97	1 1	NA NA	13.5 3.9
Vogl [100]	RFA (25) MWA (28)	R	Germany	NA NA	NA NA	1.28 1.28	72 79	9.4 8.3

Abbreviations: *CP* child-pugh; *RFA* radiofrequency ablation; *MWA* microwave ablation; *RCT* randomized controlled trial; *R* retrospective

MWA outperformed RFA in terms of LR for treatment of larger tumors (OR: 1.88, 95 %CI 1.10–3.23, $p = 0.02$) [101]. However, these findings should be interpreted with caution as this systematic review included duplicate studies conducted by the same group while did not consider two more recent non-Asian studies [99, 100] which were not available at the time of the publication of this meta-analysis [101]. Moreover, such results may not be applicable to actual series as the included studies used a previous generation MWA system while a new-generation cooled-shaft system recently became available.

Therefore, whether MWA ability to generate a larger ablation zone will translate into a survival gain remains unknown.

30.11.2 High-Intensity Focused Ultrasound Ablation

High-intensity focused ultrasound (HIFU) ablation aims to elevate tissue temperature by focusing high energy ultrasound (US) waves into one small spot [42]. The main advantage of HIFU ablation is the safety and the less invasiveness with, on the other hand, the limitation of a longer procedure time and acoustic shadowing by the rib cage, which may also cause thermal injury of the overlying soft tissue as a result of high US absorption by the bony cortex [42]. This drawback has been partially overcome by later generation systems using a larger transducer to spread the US beams out, thus reducing energy at the surface level, or a multielement phased-array transducer able to selectively activate only elements that correspond to the intercostal spaces [102]. There are actually few studies on HIFU, mainly conducted in advanced or recurrent cases for palliative purposes. Chan et al. retrospectively compared HIFU ablation and RFA for recurrent HCCs and reported no significant 3-year survival difference (69.8 % vs. 64.2 %, $p = 0.19$) [103]. The same group compared the outcomes of HIFU ablation to those of TACE as bridging therapy before OLT and found comparable percentages of tumor necrosis in excised livers ($p = 0.35$) [104]. The authors concluded that HIFU ablation was safe even for HCC patients with Child-Pugh C disease and its adoption increased the percentage of patients receiving bridging therapy from 39.2 to 80.4 % [104].

In our opinion, because of the scarce data currently available and in attendance of further reliable results in the clinical setting, HIFU represents a promising option to be performed in highly experienced centers and in selected cases.

30.11.3 Laser Ablation

Among the available ablative therapies, laser ablation (LA) is one of the least investigated.

Laser devices transform electrical energy into light energy, which interacts with tissue to produce heat and cause cell death [105]. Because laser light is coherent and monochromatic, it can be highly collimated and focused and large amounts of energy can be transmitted over long distances without significant losses. Light is delivered via multiple flexible quartz fibers which have flat or cylindrical diffusing tips. The use of water-cooled laser application sheaths enables a higher laser power output (up to 50 W compared with 5 W of previous devices) while preventing carbonization, thus allowing ablative zones of up to 80 mm diameter [106].

Several retrospective cohort studies have shown that LA is a safe and feasible procedure for the treatment of HCC with a complete response rate ranging from 82 to 97 % [106–109].

In an Italian multicenter restrospective study, 5-year cumulative survival was 41 %, median survival times were 65 and 68 months in patients with tumor size ≤ 3 and ≤ 2 cm, respectively, while median time to recurrence was 24 months [110].

In a recent RCT including 140 Milan-in patients, complete response was observed in 97.4 % of patients treated with RFA and 95.7 % with LA and mean time to local progression and overall survival were comparable between the two study groups ($p = 0.129$ and 0.693, respectively) [111]. The authors concluded that LA resulted non-inferior to RFA and therefore it should be considered as a valuable alternative for thermal ablation of small HCC in cirrhotic patients [111].

However, in spite of the apparently excellent results in terms of safety and efficacy, the low experience available worldwide currently restricts LA application to a limited number of high-volume centers.

30.11.4 Cryoablation

Cryoablation induces cytotoxicity based on cyclic applications of extremely low temperatures (−20 to −40 °C) within the tumor [42]. Multiple cryoprobes of 2–3 mm in diameter are inserted into the target lesion via a dilation catheter to ensure the rapid freezing of the nodule. Cryotherapy is delivered by means of multiple cycles and between two consecutive cycles the cryoprobes are rewarmed by a heating system.

Despite being widely used in various other cancers, the application of percutaneous cryoablation in HCC was sparsely reported. Compared to RFA, cryoablation endows several unique advantages including larger ablative zones, more clearly discernible treatment margin, less pain, and good visualization by imaging [112, 113]. However, there are also disadvantages: [1] the ablation zone of each individual probe is generally smaller than other techniques, thus requiring multiple cryoprobes applications; [2] the zone of complete lethality lies a variable distance (4–10 mm or more) inside the ice ball, therefore a larger amount of surrounding hepatic parenchyma must be frozen to ensure a sufficient safety margin; [3] there is concern over the risk of complications such as massive hemorrhage due to ice ball fracture, cold injury to adjacent organs, and cryoshock syndrome [114, 115].

Nevertheless, with the recent improvements in technology and the increasing experience acquired worldwide, cryoablation represents a promising therapeutic tool in the field of HCC ablation.

An Asian series of 866 patients within Milan criteria who underwent percutaneous cryoablation was recently analyzed: complete response was achieved in 96.1 % of patients with a major complication rate of 2.8 % and no treatment-related mortality [116]. Five-year local tumor recurrence rate was 24.2 % and 5-year survival rate was 59.5 % [116].

A recent meta-analysis including 4 retrospective studies comparing the effect of cryoablation and RFA on hepatic neoplastic lesions concluded that RFA was significantly superior in terms of safety and local recurrence [117]. However, these studies referred not only to HCC but also to other liver malignancies, used several different equipments as laparoscopic or even surgical cryoablation [117] and were mostly conducted several years ago when experience with cryoablation was still low. In a multicenter Asian RCT enrolling 360 patients with one or two HCC lesions ≤ 4 cm, cryoablation proved superior to RFA according to 3-year local tumor progression (7 % vs. 11 %, $p = 0.043$) while 5-year overall survival was similar between the two groups (40 % vs. 38 %, $P = 0.747$) [118]. Major complications occurred in seven patients (3.9 %) following cryoablation and in six patients (3.3 %) following RFA ($p = 0.776$) [118]. These results have been confirmed in an interesting retrospective study comparing cryoablation and RFA combined to microwave coagulation therapy, where hypothermal therapy proved superior to combined regimen as for 2-year local recurrence-free survival (HR 0.3, 95 %CI 0.1–0.9; $p = 0.02$) with no difference in safety outcomes [119].

Although further RCTs are needed in order to confirm these promising results, appropriate use of cryoablation could represent a valuable therapeutic option in early stage HCC patients.

30.12 Summary

Ablative treatments, particularly RFA, currently represent the first-line option for early stage unresectable HCC patients. Main indications to ablative treatments are BCLC 0/A patients not suitable to surgical therapies, namely liver resection and OLT, and bridging/downstaging setting before transplantation. Contraindication based on size, number, and location of nodules are quite variable in literature and strictly dependent on local expertise.

Among ablative therapies, RFA has gained a pivotal role due to its efficacy and safety. In fact, considering the state of art of the literature, RFA provided 5-year survival rates of 40–70 % and beyond in HCC series and, although survival rates are similar to PEI, the lower local recurrence rate stands for a wider application of RFA in hepato-oncology.

Moreover, RFA seems to be even more cost-effective than resection for very early HCC (single nodule ≤ 2 cm) and in the presence of two or three nodules ≤ 3 cm.

Prognostic factors for patient survival after RFA are rather variably reported in the literature, including CP score, initial response, serum ferritin, number or size of nodules, and AFP levels. Local recurrences (those occurring in the same segment as the primary tumor), unlike distant ones, did not prove to have a significant influence on survival, probably because they may be more easily treated.

Unlike overall survival, reported rates of local recurrence after RFA are not univocal ranging from 3.2 to 27 % at 5 years, maybe because of different baseline characteristics in the published series. On the other hand, intrahepatic distant recurrences occur very frequently, from 68 to 74 % at 5 years, and are usually associated to poorer prognosis. This type of recurrence is predominantly related to underlying hepatic disease and is often observed after 2 years, which is the time point considered able to differentiate between real recurrences from de novo tumors occurred in the pro-tumorigenic milieu of liver cirrhosis.

In the last years, a number of drugs have been tested as adjuvant treatment, such as sorafenib, ACE I and interferon, in order to decrease the high recurrence rate after RFA but no agent proved effective in this specific setting. Some promising results have been recently presented with regard to sartans but further confirmation is needed.

MWA aims to induce tumor necrosis using high frequency electromagnetic energy which generates tissue death via coagulation necrosis. In comparison to RFA, MWA has several theoretical advantages such as a broader zone of active heating, higher temperatures within the targeted area in a shorter treatment time, and it is not impaired by heat sink effect. The safety concerns raised on the risks of this procedure, due to the broader and less predictable necrosis areas, have been recently overcome. However, whether MWA ability to generate a larger ablation zone will translate into a survival gain remains unknown.

Other treatments, such as HIFU, LA, and cryoablation, are less investigated but showed promising results in early HCC patients and could be a valuable therapeutic option in the next future.

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Resection of Hepatocellular Carcinoma

31

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Contents

31.1 Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Surgery is the mainstay of HCC treatment. Liver resection and liver transplantation are considered the only curative treatment modalities, and achieve the best outcomes in properly-selected candidates, with 5-year survival of 60–80 % [1, 2]. Liver resection is accepted as the first line treatment in non-cirrhotic patients with HCC, as well as in cirrhotic patients with well-preserved liver function, and no signs of clinically significant portal hypertension. Unfortunately, the majority of patients presenting with HCC cannot undergo curative resection due to either impaired liver function, presence of portal hypertension, or tumor stage, and only 10–20 % are considered surgical candidates [3, 4]. In recent years perioperative mortality has decreased to 3–5 % in the majority of large-volume centers. This is attributed to refined surgical technique, improved patient selection, and optimization of postoperative management. The oncological principle of anatomic resection aimed to completely remove the involved segments with wide surgical margins, in cases that do not jeopardize sufficient function of the remnant liver is associated with improved outcome. Recently, the implementation of minimally invasive surgery in patients with HCC was shown to be safe and associated with decreased rates of blood transfusion, clamping time, postoperative complications, and ascites [5, 6], with similar long-term oncological results.

There is an ongoing debate regarding expanding criteria for resection in HCC patients previously considered unresectable. Current guidelines proposed by the Barcelona Clinic Liver Cancer (BCLC) group recommend resection in patients with solitary small tumors, well preserved liver function and no clinically-significant hypertension. However, the limited efficacy of alternative treatment modalities, and the improved outcomes of liver resections in high-volume centers, has caused increased interest in implementation of liver resection in highly selected patients with advanced tumor, multifocal HCC, presence of gross vascular invasion,

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or portal hypertension. The role of resection in such patients is controversial, due to increased perioperative mortality, and decreased long-term outcome. Well-designed prospective randomized trials examining the survival benefit are needed for expansion of resection criteria.

Despite improved outcome, recurrent tumor remains a major problem, and is reported in up to 75 % of patients at 5 years post resection. Recurrence may represent either metastasis from the original primary, or de novo tumor due to predisposition of the cirrhotic liver. The best therapeutic options for recurrent HCC remains poorly defined.

31.2 Indications for Resection

Liver resection is the accepted treatment for patients with early-stage HCC and normal liver function. The choice of treatment modality in patients with cirrhosis, who comprise the majority of patients with HCC, requires assessment of liver function, presence of portal hypertension, and tumor extension.

Assessment of liver function reserve is commonly done using the Child-Pugh score, and resection is accepted only in patients who are Child-Pugh class A. Bilirubin lever above 1 is generally considered a relative contraindication for liver resection. In Asia, indocyanine green retention rate at 15 min (ICG 15) is commonly used to assess liver function reserve [7, 8]. Assessment for presence of clinically significant portal hypertension can be done in several ways, including platelet count (cutoff level 100,000–150,000), presence of splenomegaly, presence of esophagogatric varices on endoscopy, or direct measurement of hepatic venous pressure gradient (HVPG, cutoff level 10 mmHg). The BCLC group has shown that 5-year survival in patients undergoing liver resection with bilirubin <1 and no evidence of portal hypertension is 70 %, compared with 50 % in patients with both risk factors [1]. Unfortunately, selection of patients with HVPG < 10 mmHg or absence of indirect signs of portal hypertension leads to resectability rate of only 10 % [4]. The impact of portal hypertension on perioperative morbidity and mortality in patients undergoing liver resection has been demonstrated in previous studies [9, 10]. However, some authors still recommend surgical resection in selected patients with portal HTN, as it may achieve better long-term outcome when compared to non-surgical treatment alternatives [11]. Prospective studies comparing surgery verses non-operative modalities in selected patients with portal hypertension are needed before expansion of the currently accepted resection criteria.

Intrahepatic tumor extension is assessed using modern axial imaging modality, either MDCT or MRI. In the guidelines of the American Association for the Study of Liver Diseases (AASLD), as well as the European Association for the Study of the Liver (EASL), resection is not recommended for patients with multifocal HCC, and patients with evidence of gross vascular invasion of large vessels [12]. However, reports from high-volume centers have shown that like in the case of portal hypertension, presence of multiple tumors or gross vascular invasion is associated with worse prognosis. Nonetheless, resection may still achieve better long-term outcome when compared to the non-surgical alternatives [11, 13]. In a recently-published randomized controlled study, patients with multifocal HCC undergoing resection had better overall 5-year survival compared with patients undergoing TACE [14].

Presence of extrahepatic hematogenous or lymphatic metastasis, main portal vein or inferior vena cava (IVC) involvement are generally accepted as absolute contraindications for liver resection.

31.3 Preoperative Assessment

Prior to selection of patients with HCC for resection, preoperative assessment is required to assure the following objectives: (1). Surgery needs to be performed with curative intent, i.e. complete tumor resection, preferably with resection of the entire tumor vascular territory, and no evidence of extrahepatic tumor extension; (2). Liver functional reserve is adequate and the likelihood of postoperative liver decompensation is low; (3). Operative risk due to general condition, nutritional status, and comorbidities is reasonably low.

31.3.1 Evaluation of Tumor Extent

Lung metastases need to be ruled out with chest CT. Abdominal CT is used to evaluate for presence of enlarged metastatic lymph nodes, adrenal metastases, or peritoneal spread. Some centers recommend routine Technetium bone scan to rule out bony metastases, although in asymptomatic patients the yield is low. Symptoms suggestive of bony or brain metastases should be thoroughly evaluated. FDG-PET scans have a sensitivity of 76 % in detecting extrahepatic metastases in patients with HCC, with higher yield in those with advanced or high grade tumors [15, 16].

Assessment of extent of tumor burden including location, size, number of lesions, and proximity to major structures, is done with either MDCT, or contrast-enhanced MRI. The newer hepatocyte-specific contrast agents, such as gadolinium ethoxybenzyl diethylenetriemine pentaacetic acid (Gd-EOB-DTPA), have shown increased sensitivity and specificity in the diagnosis, and detection of additional tumor nodules, in patients with HCC [17]. The appearance of typical hyperintense lesion on arterial phase, followed by washout on the portal phase, and hypointensity on the

delayed hepatobiliary phase, are diagnostic for HCC in the presence of an underlying liver disease, and obviate the need for preoperative biopsy, which carries a small but definite risk for tumor rupture, bleeding and spread.

Tumor size is not a contraindication for liver resection, and reasonable short and long-term outcomes have been reported in patients undergoing resection for tumors larger than 10 cm [13, 18]. HCC invasion of major portal, hepatic venous, and biliary structures needs to be assessed. Patients with gross involvement of vascular or biliary structures have a very limited life expectancy, in the range of 3–4 months, and are classified by the BCLC staging system as stage C. The recommended treatment for such patients is sorafenib, and these patients have a median survival of 8 months [19]. Several series reported results of TACE for HCC with gross vascular invasion. The median survival in these studies ranged between 4 and 6 months [20, 21]. Multiple studies have reported the outcome of liver resection in such circumstances, with median survival of 6–20 months, and 5-year survival of 0–40 % [22–25]. Generally, invasion of main portal vein, IVC, or common bile duct are considered a contraindication for surgery, while involvement of such structures within the liver segment or lobe to be resected is not.

Patients with more than one tumor nodule have a very high likelihood of recurrence following resection. Patients that have a limited tumor burden, within the Milan criteria should be considered for liver transplantation. Patients that are beyond Milan criteria, are classified as stage B according to the BCLC staging system, and the recommended treatment for these patients is TACE. However, there are multiple retrospective studies reporting results of liver resection for multifocal HCC, with survival rates that are better than those reported following TACE [11, 13]. A recent randomized, controlled study has shown that liver resection offers better long-term outcome compared to TACE in patients with multifocal HCC outside the Milan criteria [14], therefore liver resection is a valid option in this setting provided complete tumor removal can be safely performed.

31.3.2 Evaluation of Liver Function Reserve

This involves determining the functional residual liver volume after resection, assessment of liver function, and severity of portal hypertension.

Future Liver Remnant (FLR) can be calculated using volumetry based on high resolution CT. In normal livers, FLR as low as 20 % can be tolerated. In patients with liver fibrosis or cirrhosis, higher volumes are needed, and FLR of 40 % is considered the lower limit in Child A cirrhosis. Functional liver volume needs to be estimated, by subtracting the tumor volume from the total liver volume. This is important mainly in patients with bulky tumors. Patients with smaller tumors, in whom the resected liver is largely made of normal parenchyma, have a higher likelihood of developing postoperative liver failure, when compared to patients with large tumors, in whom the resected liver is mostly non-functioning tumor tissue.

Liver function status can be assessed using clinical staging systems such as the CTP score. Resection is generally considered only in patients with CTP A cirrhosis. Patients with CTP B or C do not tolerate resection well, and should be considered for either liver transplantation, or alternative non-surgical treatment modalities, such as loco-regional therapies or medical therapy. Recently, the MELD score has also been shown to correlate with the risk of postoperative liver failure, and most studies report higher risk in patients with a MELD score of >=9 [26, 27]. In Asia, indocyanine green retention at 15 min (ICG15) is used to measure liver function. A normal cutoff value predictive of safe major and minor resections are 15 and 22 %, respectively [28, 29]. However, it should be remembered, especially when planning major resections, that this test does not provide information on the relative function of the FLR.

Preoperative portal vein embolization (PVE) is a well-described method for preoperative modulation of liver volumes in patients with small FLR. Multiple studies have shown improved perioperative outcomes in patients undergoing major hepatic resections for HCC following PVE [31, 32]. However, patients with liver cirrhosis often have reduced capacity for hypertrophy following PVE, compared to normal livers. In a way, PVE can be used as a ‘stress test’ to evaluate the liver regenerative capacity. Absence of early hypertrophy following PVE is considered a failed ‘stress test’, and a relative contraindication for liver resection [33]. Additional, less conventional options to improve regeneration of the FLR include sequential TACE-PVE [34], hepatic vein embolization [35], and high dose lobar radioembolization using Y90 spheres [36].

Presence of significant portal hypertension is an extremely important variable in determining risk of surgical resection. Presence and severity of portal hypertension can be assessed using direct, and indirect measures. Presence of esophagogastric varices on upper endoscopy, collaterals and enlarged spleen on cross sectional imaging, and thrombocytopenia (cutoff level, 100,000/μl) are indirect signs of clinically significant portal hypertension. The gold standard for assessing portal hypertension is HPVG, a measure of the pressure gradient between the wedged hepatic venous pressure, which estimates the portal pressure, and the free hepatic venous pressure. A pressure gradient above 10 mmHg is considered portal hypertension, and is associated with poor outcomes following liver resection [10].

31.3.3 Evaluation of Operative Risk

Additional factors that need to be evaluated and determine the ability of the patient to tolerate major surgery include age, general functional status, nutritional status, and presence of comorbidities. Specifically, age > 70, American Society of Anaesthesiologist (ASA) score > 3, and chronic renal failure, are considered significant risk factors in patients undergoing liver resection for HCC [37, 38].

31.4 Principles of Surgical Resection

There has been significant improvement in the perioperative results following liver resection, mainly due to techniques that help reduce blood loss during the operation. Liver resection for HCC should be performed in high-volume centers. Such centers should be capable of offering the full scope of treatment modalities for patients with HCC, including liver transplantation, percutaneous ablative modalities, and transarterial therapies.

Extent of liver resection required in HCC for optimal oncologic results is still controversial. HCC has a well-described propensity to invade portal structures and send metastases via the portal circulation. Indeed, most recurrences following liver resection for HCC occur in the liver, and presence of both microvascular, and macrovascular portal invasion were shown to be powerful predictors of both recurrence and survival [39–41]. On this basis, the rationale for anatomically removing the entire segment or lobe bearing the tumor, would be to remove undetectable tumor metastases along with the primary tumor. The main concern with anatomic resection, specifically in patients with impaired liver function, is that removal of a significant portion of functional liver tissue would result in postoperative liver failure. Several retrospective studies and meta-analyses have shown that anatomical resections are safe in patients with HCC and liver dysfunction, and may offer a survival benefit [42, 43]. It should be noted, that most studies are biased, as non-anatomical resections are more commonly performed in patients with more advanced liver disease, which affects both recurrence and survival. It therefore remains unclear whether anatomical resections have a true long-term survival benefit in patients with HCC. Some authors have suggested that anatomical resections may provide a survival benefit in tumors between 2 and 5 cm [44]. The rational is that smaller tumors rarely involve portal structures, and in larger tumors presence of macrovascular invasion and satellite nodules would offset the effect of aggressive surgical approach. Another important predictor of local recurrence is margin status. Generally, a tumor-free margin of 1 cm is considered necessary for optimal oncologic results. A prospective randomized trial on 169 patients with solitary HCC demonstrated that a resection margin aiming at 2 cm, safely decreased recurrence rate and improved long-term survival, when compared to a resection margin aiming at 1 cm [45]. Therefore, wide resection margins of 2 cm is recommended, provided patient safety is not compromised.

Intraoperative ultrasound (IOUS) is an extremely important tool when performing liver resections, specifically for patients with HCC and compromised liver function. IOUS allows for localization of the primary tumor, detection of additional tumors, satellite nodules, tumor thrombus, and define relationship with bilio-vascular structures within the liver. Contrast-enhanced ultrasound, used mainly in eastern countries, provides additional information mainly on small nodules. Finally, intraoperative US-guided injection of dye, such as methylene-blue, to portal branches can clearly define the margins of the segment supplied by the portal branch and facilitate safe anatomical resection.

The anterior approach to liver resection is a technique aimed at limiting tumor manipulation to avoid tumoral dissemination, decrease potential for blood loss caused by avulsion of hepatic veins, and decrease ischemia of the remnant liver caused by rotation of the hepatoduodenal ligament [46, 47]. This technique is described for large HCCs located in the right lobe, and was shown in a prospective, randomized trial to reduce frequency of massive bleeding, number of patients requiring blood transfusions, and improve overall survival in this setting [47]. This approach can be challenging, and can be facilitated by the use of the hanging maneuver [48].

Multiple studies have demonstrated that blood loss and blood transfusion administration are significantly associated with both short-term perioperative, and long-term oncological results in patients undergoing resection for HCC [49]. This has led surgeons to focus on limiting operative blood loss as a major objective in liver resection. Transfusion rates of <20 % are expected in most experienced liver surgery centers. Inflow occlusion, by the use of the Pringle maneuver represents the most commonly performed method to limit blood loss. Cirrhotic patients can tolerate total clamping time of up to 90 min, and the benefit of reduced blood loss outweighs the risks of inflow occlusion, as long as ischemia periods of 15 min are separated by at least 5 min of reperfusion [50]. Total ischemia time of above 120 min may be associated with postoperative liver dysfunction [51]. Additional techniques aimed at reducing blood loss include total vascular isolation, by occluding the inferior vena cava (IVC) above and below the liver, however, the hemodynamic results of IVC occlusion may be significant, and this technique has a role mainly in tumors that are adjacent to the IVC or hepatic veins. Anesthesiologists need to assure central venous pressure is low (below 5 mmHg) by limiting fluid administration, and use of diuretics, even at the expense

of low systemic pressure and use of inotropes. After completion of the resection, large amount of crystalloids can be administered to replenish losses during parenchymal dissection.

In recent years, there had been significant advances with the use of laparoscopy for liver resections. Laparoscopic liver resections were shown to provide benefits of reduced surgical trauma, including a reduction in postoperative pain, incision-related morbidity, and shorten hospital stay. Some studies have demonstrated reduced operative bleeding with laparoscopy, attributed to the increased intra-abdominal pressure which reduces bleeding from the low-pressured hepatic veins [52, 53]. Additional potential benefits include a decrease in postoperative ascites and ascites-related wound complications, and fewer postoperative adhesions, which may be important in patients undergoing salvage liver transplantation. There has been a delay with the use of laparoscopy in the setting of liver cirrhosis, due to difficulties with hemostasis in the resection planes, and concerns for possible reduction of portal flow secondary to increased intraabdominal pressure. However, several recent studies have suggested that laparoscopic resection of HCC in patients with cirrhosis is safe and provides improved outcomes when compared to open resections [53, 54]. Resections of small HCCs in anterior or left lateral segments are most amenable for laparoscopic resections. Larger resections, and resection of posterior-sector tumors are more challenging and should only be performed by very experienced surgeons. Long-term oncological outcomes of laparoscopic resections was shown to be equivalent to open resections on retrospective studies [53, 55], but prospective studies are needed to confirm these findings. In recent years, robotic-assisted liver resections are being explored (*). Feasibility and safety of robotic-assisted surgery for HCC has been demonstrated in small non-randomized studies [56], but more experience is needed, and long-term oncologic results need to be studied, before widespread use of this technique will be recommended.

As noted in the previous part of this chapter, one of the main limiting factors to major liver resections in patients with HCC is the amount and quality of the FLR. The pre-operative options for inducing atrophy of the resected part and hypertrophy of the FLR, mainly PVE, were described earlier. Associating Liver Partition with Portal vein ligation for Staged hepatectomy (ALPPS) is another surgical option aimed to induce rapid hypertrophy of the FLR in patients with HCC. This technique involves a 2-stage procedure. In the first stage splitting of the liver along the resection plane and ligation of the portal vein is performed, and in the second stage, performed at least 2 weeks following the first stage, completion of the resection is performed. Patient safety is a major concern, and some studies have reported increased morbidity and mortality with the procedure. Few reports exist of this procedure in the setting of liver cirrhosis [57]. Currently, the role of ALPPS in the setting of HCC and liver dysfunction needs to be better delineated before more widespread use is recommended.

Another strategy available for patients with multifocal HCC is combined resection and radiofrequency or microwave ablation (RFA or MWA). Resection of a large tumor, not amenable to ablative treatments, and ablation of additional small tumors in the FLR can be performed safely. This can allow complete local control of the liver tumors, and preservation of sufficient liver parenchyma thereby maintaining patient safety [58].

31.5 Results of Liver Resection

31.5.1 Perioperative Outcomes

The perioperative mortality of liver resections for HCC has decreased significantly in past years from 15 % in the 1980s to less than 5 % in most of the large liver centers, with some reporting mortality <1 % [59]. As discussed previously, predictors of perioperative mortality are mainly related to the degree of liver dysfunction, presence of portal hypertension, and extent of resection.

Amount of blood loss and requirements for blood transfusion administration have repeatedly been reported to correlate with both increased perioperative mortality, and poor long-term outcome. Techniques aimed at reducing operative blood loss are discussed above, and probably have an important role in the reduced operative mortality and improved long-term outcome reported in recent years. Currently, rate of blood transfusion is less than 10 % in most reports.

Overall, and major morbidity rates following liver resection for HCC are as high as 60 and 30 %, respectively [60, 61], and higher than those observed after liver resection for other pathologies, such as colorectal liver metastases. Most frequent complications include ascites, pulmonary complication such as pleural effusion and pneumonia, and bile leak. Less common, but life threatening complications include liver failure, bleeding, and portal vein thrombosis (PVT). Postoperative liver failure (PLF) is defined as the inability of the remaining liver parenchyma to maintain adequate function. PLF is often accompanied, and exacerbated by sepsis. Aggressive workup for identification and treatment of the source of infection, such as an infected biloma, infected ascites, or extra-abdominal sites, is crucial. Postoperative bleeding requiring re-laparotomy for hemostasis is a rare, but life-threatening complication, with associated mortality of up to 40 % [62]. Perfect hemostasis needs to be assured at the completion of the hepatectomy to prevent this serious and avoidable complication. Use of

fibrin sealant agents on the cut-surface of the liver has been introduced in recent years, and can lead to improved hemostasis [63]. Postoperative PVT may occur in up to 3 % following liver resection [64]. Risk factors for development of PVT include large resections, and prolonged inflow occlusion [64]. Clinical signs are variable, ranging from asymptomatic incidental finding, to rapid deterioration of liver function and mortality. Early diagnosis with routine postoperative ultrasound-Doppler is advocated by some centers. Treatment options include administration of anti-coagulants, and surgical thrombectomy.

31.5.2 Long-Term Results

Recurrence of HCC after resection is common, and develops in up to 85 % of patients at 5 years post resection [1, 65]. By far, the most common site of recurrence is the liver, and may be the result of either metastases from the original tumor, or de novo tumors related to the underlying liver disease. Distinguishing between metastases and de novo disease may have important prognostic and clinical implications, specifically regarding eligibility for salvage liver transplantation, and can theoretically be done using molecular markers. Practically, as most true recurrences appear within 2 years of the resection, this has been adopted as the cut-off to distinguish true tumor recurrence from new tumor development. Predictors of true HCC recurrence include presence of vascular invasion, tumor grade, tumor size, number of tumors, presence of satellites, alpha-feto protein level, administration of blood transfusion during the operation, type of surgical resection (anatomic verses nonanatomic), and surgical margin status. Presence of vascular invasion is probably one of the most important tumor-related features predicting both survival and recurrence. Degree of vascular invasion is also important, and gross vascular invasion is a stronger predictor than microscopic vascular invasion. Pathological features of microscopic vascular invasion used to grade the degree of vascular involvement, such as the number of cells within the invaded vein, distance between the invaded vessel and the tumor nodule, and size of the invaded vessel, also correlate with recurrence and survival post resection [41]. Features that are associated with de novo tumor development following resection of HCC include degree of liver fibrosis, and etiology of liver disease (HCV more than HBV- 66).

Postresection overall survival rates are influenced mostly by operative mortality, tumor recurrence, and progression of the underlying liver disease. The ability to control progression of liver disease, for example by the use of antiviral medications in the case of HBV and HCV, can improve long-term survival post resection [65, 67, 68]. Reported survival rates range from 80–90 % at 1 year, 60–85 % at 3 years, and 40–75 % at 5 years post resection [1, 65, 69, 70]. Ten-year survival after liver resection for HCC can be expected in approximately 15 % of patients [71].

31.6 Adjuvant Treatment

The value of several adjuvant therapies aimed at reducing recurrence following liver resection were explored. The heterogeneity of patients that need to be evaluated in the adjuvant setting due to variability of etiology of liver disease, degree of liver dysfunction, and tumor characteristics, renders the designing of randomized controlled studies that can be used to promote clear conclusions challenging. Several trials have looked at the value of modalities including preoperative [72] and postoperative TACE [73], as well as chemotherapy [74], and these were not shown to improve survival. The recently reported STORM trial demonstrated in a placebo controlled randomized trial that sorafenib is not effective in the adjuvant setting for HCC following resection or ablation [75].

The only adjuvant therapy of proven value is treatment of the underlying liver disease. In patients with HBV related liver disease, use of antiviral treatment with nucleoside analogues has been shown to halt progression of liver disease, and reduce development of new HCCs [65, 67]. Similarly, treatment of HCV with interferon-based therapies following liver resection was proved to reduce recurrence of HCC [68]. This effect is likely to be greater with the use of the newer HCV medications.

31.7 Treatment of Tumor Recurrence

As discussed previously, recurrence of HCC after liver resection remains a significant problem. Recurrence rate after resection approaches 80 % at five years, and in 65–80 % of cases the liver is the only site of disease [76]. Potentially curative treatments in patients with liver-only recurrence following resection include a second resection, liver transplantation, and percutaneous ablation. Repeat resection is currently considered the treatment of choice in patients with resectable disease, preserved liver function, and no signs of portal hypertension. Thus, only 15–20 % of patients with recurrence are considered candidates for repeat resection [77, 78]. Oncologic outcome following repeat resection for HCC recurrence in well-selected patients is reasonable, with reported 5-year survival of 31–67 % [77, 79–81]. Short disease-free interval between resection of the primary tumor and diagnosis of recurrence, as well as presence of macroscopic vascular invasion on the second hepatectomy were identified as poor prognostic factors in patients undergoing repeat resection. Up to 60 % of patients with HCC recurrence following resection are within the

Milan Criteria and are therefore eligible for liver transplantation [82]. Transplantation in the setting of previous liver resection can be challenging, especially in patients with significant portal hypertension. 5-year survival after such 'salvage transplantation' was 70 % [82].

In patients with unresectable disease that are beyond the Milan criteria, TACE is the most widely used treatment modality. Results are comparable to those reported in BCLC stage B patients. Management of extrahepatic recurrence after hepatectomy is not well studied. Small retrospective studies suggest that selected patients with limited extrahepatic disease may benefit from aggressive surgical treatment [83].

31.8 Conclusions

Improvements in surgical technique, patient selection, and perioperative care has resulted in significant improvements in perioperative and long term outcomes in patients undergoing liver resection for HCC. Liver resection is currently considered the first-line treatment in patients with single tumors and normal liver function, as well as patients with CTP A cirrhosis and no portal hypertension. With the dramatic improvements in perioperative outcomes, and lack of better therapeutic options, surgery is also considered in more controversial scenarios, such as multifocal HCC, gross vascular invasion, and presence of portal hypertension. Minimal invasive techniques, such as laparoscopy, and robotic-assisted liver resections are feasible and have the potential to further reduce morbidity and expand resection criteria. The major factors limiting long-term survival following liver resection for HCC are progression of liver disease, and recurrence of the primary tumor. The only adjuvant treatments shown to improve long-term survival and reduce recurrence are those that deal with the etiology of liver disease, e.g. treatment of hepatitis B or C. In cases of recurrence limited to the liver, repeat resection and salvage liver transplantation are valid therapeutic options.

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Liver Transplantation for Hepatocellular Carcinoma

32

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Abbreviations

AFP	Alpha-fetoprotein
AFP-L3	Lens culinaris agglutinin A-reactive fraction of alpha fetoprotein
CNI	Calcineurin inhibitor
DCP	Des-gamma-carboxy prothrombin
DDLT	Deceased donor liver transplantation
HCC	Hepatocellular carcinoma
LDLT	Living donor liver transplantation
LT	Liver transplantation
LRT	Locoregional tumor treatment
MC	Milan criteria
MiVI	Microvascular invasion
m-TOR	Mammalian target of rapamycin
RFA	Radiofrequency ablation
SRL	Sirolimus
TACE	Transarterial chemoembolization
TTV	Total tumor volume
UCSF	University of California San Francisco
VEGF	Vascular endothelial growth factor

Contents

Hepatocellular carcinoma (HCC) is a major health problem worldwide because of the association of HCC with chronic liver injury and inflammation due to viral, non-viral and genetic etiologies [1]. While historically HCC was often considered for palliative therapy, new curative alternatives have emerged such as liver resection, loco-regional therapies and liver transplantation (LT). With respect to any other available treatment, LT has the highest potential to cure both the seeded tumor and the underlying liver disease at once. Prior to 1996, constraints on LT for HCC were more liberal which resulted in disappointing high recurrence rates (>50 %) and discouraging 5-year overall survival results ranging from 10 to 35 % [2]. Since it appeared obvious that the success of LT for HCC depends on tumor load, strict selection criteria were introduced with regard to size and number of tumor nodules (Milan criteria (MC): any solitary HCC ≤ 5 cm or up to 3 cm each, without macrovascular invasion or metastasis) [3]. These criteria resulted in 5-year transplant survival near 70 % with recurrence appearing in less than 10 %. These post-transplant survival outcomes match those from most other liver transplant indications. Hence, the last 2010 international consensus conference recommended using the Milan criteria as the benchmark not only for selecting HCC patients for LT, but also for future comparisons of expanded selection criteria and refinements [4].

This evolution is mainly due to improvement of imaging techniques and surveillance programs, which have been widely introduced. As a result HCCs are being detected earlier at a stage at which effective treatment is feasible. In this context, LT for HCC currently represents 20–30 % of

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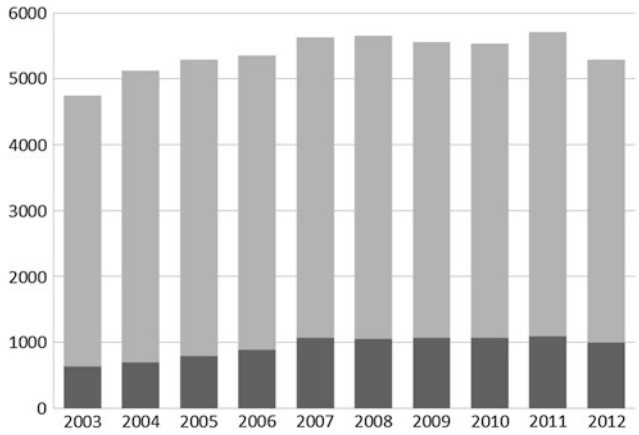

Fig. 32.1 Total amount of liver transplantations (*light grey*) from 2003 to 2012 in Europe in comparison to liver transplantations for HCC (*dark grey*) from the same area. Based on European Liver Transplantation Registry (ELTR) data as of October 31, 2015

the indications for LT in Europe and in the US, respectively [5, 6] (Fig. 32.1). The need to obtain the optimal benefit from the limited number of organs that are available, has prompted the maintenance of selection criteria in order to list only those patients with early HCC who have the highest likelihood to survive after LT. However the indications for LT and organ allocation system led to many controversies around the use of LT in HCC patients.

The aim of this chapter is to give an updated overview about developments of LT for HCC focusing on selection criteria, prognostic factors, treatment on the waiting list, role of living donor liver transplantation (LDLT), adjuvant therapy and impact of immunosuppression on HCC recurrence after LT.

32.1 How Far Can the Selection Criteria Be Extended?

The possibility of extending selection criteria for transplantation has to be seen as a triangulation of organ availability, waiting list survival and recipient outcome after liver transplantation. Shortage of donor organs can also prolong the time on the waiting list, increasing the risk of waiting list dropout due to tumor progression.

According to studies based on Markov models using data from the USA [7], patients listed for transplantation with HCC would need to achieve 5-year survival of 60 % or higher to prevent a substantial decrement to the life-years available to the entire population of candidates for liver transplantation. Any decision by a center to expand criteria should take into account the current mortality on the waiting list, and should only be done if a low mortality will not be substantially increased by additional expanded criteria cases [4].

Thus to this day the Milan criteria (single HCC nodule of <5 cm or up to three nodules of <3 cm without macrovascular invasion) are the benchmark for selecting patients with HCC to be listed for liver transplantation [3]. Within these criteria excellent 5-year survivals up to 78 % have been reported [8]. Recent studies however report comparable results in patients beyond the Milan criteria. The group from UCSF—one tumor <6.5 cm, or two or three nodules <4.5 cm with total tumor diameter <8 cm—demonstrated a tumor recurrence rate of 11 % with a comparable 5-year survival rate of patients displaying T1/T2 tumors (72 %) and those with T3 tumors (74 %) [9].

Over a period of 5 years, they prospectively validated these results based on pre-transplant imaging in a cohort of 168 patients, including 38 patients with HCC exceeding the Milan criteria [10]. The 1-and 5-year recurrence-free probabilities were 96 and 91 % and the survival without recurrence was 92 and 81 %, respectively. Other studies including transplanted HCC patients within the UCSF criteria achieved comparable outcomes [11, 12]. Noteworthy, except for one study where 40 % (n = 185) of HCC patients were outside the Milan criteria but within the UCSF criteria [11], the use of the latter criteria resulted in only a modest expansion of the number of eligible patients by 5–10 %.

Asan Medical Centre in Seoul, South Korea also could show comparable 5-year overall survival of 76 % within their Asan criteria—largest tumor ≤ 5 cm, number of nodules ≤ 6, and no gross vascular invasion—in a population mainly transplanted from living donors [13]. A recent analysis comparing Milan, UCSF and Asan criteria in a long-term follow up found that expansion of eligible patients was somewhat higher compared to UCSF when using Asan criteria (26 % vs. 15 %). After a median follow-up of 70 months, patients exceeding MC but fulfilling Asan criteria had comparable 5-year overall survival and disease-free survival to patients fulfilling MC ($p = 0.17$; $p = 0.29$). Patients exceeding UCSF but fulfilling Asan criteria had comparable 5-year overall survival and disease-free survival to patients fulfilling UCSF criteria ($p = 0.26$; $p = 0.32$). Number of nodules, macrovascular invasion, capsular invasion, and exceeding Asan criteria predicted recurrence in multivariate analysis [14].

The connection between size and number of tumors as well as presence of vascular invasion has been well described in the "Metroticket concept" (the farther you go in expansion of HCC staging criteria for selection for LT, the more you have to pay in terms of higher recurrence rates and poorer survival) [15]. This model, based on the analysis of 1556 patients transplanted at 36 centers, provides a linear correlation between tumor diameter and recurrence throughout the observed range. The survival was directly correlated with the size of the largest tumor, number of tumors and presence of microvascular invasion (MiVI) at

explant pathology examination. Patients who were within the "up to 7 criteria" (HCC with 7 as the sum of the largest tumor diameter in cm and number of tumors and without MiVI), achieved a 5-year overall survival of 71 %. These "up to 7 criteria" were compared with Milan and UCSF criteria in a pathological study [16]. "The Metroticket" performed the best as a staging system with a 5-year recurrence rate of 4 % in patients within and 51 % in patients beyond those "up to 7 criteria". However, this staging system is difficult to use in practice since the MiVI cannot be accurately assessed by any preoperative work-up.

Based on the current literature presented here an expansion of selection criteria beyond Milan is feasible under careful consideration of available donors, center specific wait list mortality and postoperative outcomes.

32.2 Which Biomarkers Should Be Considered for Clinical Decision?

Measurement of tumor biomarkers is an important tool for clinical management in HCC patients. Besides alpha-fetoprotein (AFP), Lens culinaris agglutinin A-reactive fraction of alpha fetoprotein (AFP-L3) and des-gamma-carboxy prothrombin (DCP) have been established as HCC specific tumor markers [17]. While the association between tumor size and vascular invasion seems established, currently incorporation of biological tumor markers into organ allocation is under debate and well-defined cut-offs are being assessed [18]. Merani et al. showed in a cohort from the US including 6817 HCC patients listed for LT that patients downstaged to AFP values $\leq$ 400 ng/mL immediately before LT showed better intent-to-treat survivals compared to cases in which their values could not be reduced (81 % vs. 48 % 3-year overall survival; $p < 0.001$) [19]. The results were also comparable to those patients having stable AFP values $\leq$ 400 ng/mL (74 %; $p = 0.14$). Further they found that only the last pre-transplant AFP independently predicted survival. Another group from Canada defined a composite score combining tumor volume and AFP. Patients with a total tumor volume (TTV) >115 cm^3 and AFP >400 ng/mL showed survivals inferior to 50 % at 3-years after liver transplantation [20]. When compared to the Milan and UCSF criteria, the combined TTV/AFP score provided the best prediction of outcome.

Chinese and Italian groups associated tumor diameter larger than 8 cm and a cut-off of AFP >400 ng/mL to inferior survival [21, 22]. A recent analysis from the United Network for Organ Sharing in patients undergoing locoregional therapy before LT showed that peak AFP value >400 ng/mL and AFP at LT >400 ng/mL were associated with poor outcomes [23]. Despite the common theme, a variety of smaller series suggest lower AFP cut-offs between 100 and 300 ng/mL as a predictive factor for oncological outcomes after LT [24–26]. Others found that dynamic changes in AFP levels of >15 ng/mL/month while waiting for LT is the most relevant preoperative predictor of recurrence and overall survival after OLT [27].

Duvoux et al. recently proposed a model incorporating tumor size and AFP in a large retrospective series [28]. This study is particularly interesting as they were able to show that in a group outside MC patients with AFP <100 ng/mL had a very low 5-year risk of recurrence rate at 14 % vs. 48 % in the group with AFP >100 ng/mL. Further in the group within MC, patients with AFP levels >1000 ng/mL were exposed to high risk of recurrence (37 %). They conclude that addition of AFP improves the assessment of eligible candidates for LT. Similar results were described by Hameed et al. in a cohort of 211 patients within MC where AFP >1000 ng/mL was significantly associated with a higher recurrence rate after 5 years and with vascular invasion [29].

A very recent analysis of 313 patients from the Mayo Clinic concluded that AFP >250 ng/mL and DCP >7.5 ng/mL were associated with a 5 fold risk for tumor recurrence [30]. This strong association between AFP and DCP was also shown in a Japanese series of 124 LDLT recipients as well as in patients undergoing liver resection for HCC [31, 32].

While there is some evidence that assessment of (one or more) biological markers might improve allocation, biomarkers other than AFP are not yet used for clinical decision making regarding liver transplantation for HCC [4]. More information is needed to define specific cut-offs.

32.3 Do Patients Benefit from Bridging Therapies on the Waiting List?

Bridging strategies are defined as locoregional tumor treatment (LRT) in patients on the waiting list until they receive a graft [33]. Pretransplant LRTs include transarterial modalities (transarterial chemoembolization (TACE), transarterial radioembolization), percutaneous thermal ablative strategies (radio frequency ablation (RFA), microwave ablation) and surgery and have been widely adopted by transplant programs to bridge and/or downstage HCC recipients before LT [34]. LRTs are effective by achieving pathologic tumor necrosis, with reported rates of complete response in up 60 % of patients after TACE [35, 36] and up to 75 % after ablative regimens as RFA [37–39]. Although LRT does reduce the risks of tumor progression and dropout, [35–40] data on its effectiveness in reducing posttransplant HCC recurrence and improving posttransplant survival are limited and controversial [41–45].

A retrospective case control study investigated the results of TACE on outcome after LT [45]. In this study, there was no difference in the 5-year survival rate (69 % with TACE vs. 64 % without TACE) but recurrence was less frequent after TACE (13 % vs. 23 %). A small single center study on 104 patients from the US was able to show that the absolute number of TACE treatments was not associated with survival. Interestingly patients with only a single TACE application were more likely to show recurrence. They concluded that when feasible TACE application can be repeated during waiting time [44].

In pathological studies, the results of RFA appear to be superior to TACE in terms of local tumor control [39, 46]. Mazzaferro et al. showed in patients who underwent RFA as a bridge treatment to LT, that tumor size >3 cm or the presence of large abutting vessels results in a decrease in the rate of complete tumor necrosis to 50 % or less [38]. RFA appears then to be safe as a bridging therapy for HCC less than 3 cm in size. However, its ability to decrease the dropout rates still needs to be proven in further prospective trials.

Radioembolization represents 5–10 % of bridging LRT in the organ procurement and transplantation network registry, but data available on its impact are scarce and further experience is needed [33]. In a retrospective analysis looking at the radiopathological effects on HCC treated with internal radiation using yttrium-90 microspheres, all targeted lesions had some histologic necrosis and 60 % of them showed complete necrosis [46]. A recent large single center series of 501 patients listed and transplanted for HCC in the US found that no viable tumor on last examination, lowering in AFP between LRT and LT, labMELD decrease and longer time from LRT to LT were predictive factors for complete pathological response. Most interesting in this context was that complete pathological response to LRT was a strong predictor for recurrence-free survival [47].

In HCC patients with compensated cirrhosis listed in centers with an expected waiting time longer than 1 year, tumor resection followed by listing for LT could be an option [33, 48]. Careful assessment of liver function as well as size and location of the tumor determine the feasibility of a surgical option. Cherqui et al. showed that liver resection for small solitary HCC in compensated cirrhosis yields an overall survival rate comparable to LT. Despite the high significant recurrence rate, close surveillance after liver resection allows salvage LT in two thirds of the patients with recurrence in intent-to-treat analysis [49]. Fuks et al. evaluated liver resection for HCC as first-line treatment in transplantable patients within MC followed by salvage LT in case of recurrence comparing them to a group of patients within MC who underwent primary liver transplantation [50]. In both groups, 5-year overall and disease-free survivals were similar (60 % vs. 77 % and 56 % vs. 40 %, respectively). The predictive factors for dropout due to recurrence beyond Milan criteria after liver resection included microvascular invasion, satellite nodules, tumor size >3 cm, poor differentiation, and liver cirrhosis. It can be concluded that salvage LT should be restricted to patients with favorable oncological factors found on the specimen of liver resection. In other words, in case of poor prognostic factors (poor differentiation, MiVI, absence of capsule), a pre-emptive LT could be advised (i.e. before recurrence but after sufficient observation). If the tumor does not show any risk factors for recurrence, LT may be postponed and offered only in cases of tumor recurrence (salvage procedure).

Bridging strategies with locoregional treatments are beneficial in patients when a long waiting time is likely because it decreases dropout rates without impairing post-transplant outcomes. If successful, post-transplant recurrence-free survival has been shown to be significantly higher [47]. Overall bridging therapy seems to be indicated for T2 tumors (solitary tumor with vascular invasion or multiple tumors none more than 5 cm) and patients likely to wait longer than 6 months. Pathological studies suggest that there is a marginal advantage for RFA in terms of local ablation [4, 39].

Newer strategies combining TACE and RFA or using yttrium-90 may be promising. Finally, liver resection followed by salvage LT in case of recurrence should be restricted to patients with favorable oncological findings.

32.4 Role of Downstaging Before LT

Tumor downstaging is a process involving expanded criteria for listing and the effects of LRT. Per definition LRT is specifically used to meet acceptable criteria for transplantation. Rather than altering tumor biology it serves as a tool to identify patients with a high probability for beneficial outcomes [51]. There has been consensus that current goal for downstaging patients should be equally effective as patients without downstaging [4]. The literature reports successful downstaging rates of up to 90 % with significant heterogeneity in protocols [52].

At the moment, there is no well-defined upper limit for size and number of lesions as eligibility criteria for downstaging, although the presence of macrovascular invasion and extrahepatic disease are generally considered absolute contraindications [4]. The UCSF group recently published their long-term results on 118 patients downstaged to within Milan [53]. While there is a higher dropout rate at 1 year in the downstaged group (24 % vs. 20 %), in multivariate analysis factors predicting drop out were AFP >1000 ng/mL and Child B cirrhosis but not tumor size or number. After LT 5-year overall survival in the downstaged group was 78 % (vs. 81 %), meeting the recommendation of the last international consensus conference [4].

This study was also included in a recent systematic review and pooled analysis of thirteen studies (11 retrospective and 2 prospective) including 950 patients [54]. Overall downstaging rate in this study was 54 % and overall HCC recurrence rate was 16 %. No difference between TACE and radio-embolization with Yttrium 90 was reported. Of note, the success of downstaging was higher in the prospectively conducted studies, most likely related to stricter patient selection, mandatory waiting time and higher consistency in the downstaging procedures. The authors could not aggregate post-LT survival due to the inconsistent assessment of outcome criteria and further limitations reporting inclusion criteria and downstaging protocols.

There is still a debate about how best to assess successful downstaging. The European Association for the Study of the Liver (EASL) guidelines suggest that such assessment should be exclusively based on the amount of viable tumor, as differentiated from necrosis by contrast CT or MRI [55]. Most available reports have used the Milan criteria to define successful downstaging [53, 56–58], with few data available on downstaging to "up-to-seven criteria" [59]. Some groups also combine serum AFP levels for assessment of downstaging, [23] comparable data however are scarce [52, 54].

In the future, hepatic resection may play a more predominant role in downstaging as well as in curative approach since fewer patients progress to cirrhosis due to newly available antiviral drugs for HCV [52].

Downstaging HCC in the setting of liver transplantation seems feasible. Standardized methods and data assessment across studies are needed in order to ultimately determine the place of downstaging. Furthermore studies are mandatory to address whether there is an upper limit for lesions and whether liver resections may have a role in downstaging.

32.5 What Is the Role of Living Donor Liver Transplantation for HCC?

As the need for donor livers exceeds organ availability in most countries, living donor liver transplantation (LDLT) has been suggested as an alternative to organ shortage and increasing waiting lists [60]. In this setting, donor safety is a priority knowing that the incidence of operative mortality and morbidity ranges between 0.15–0.50 % and 30–40 %, respectively when using the right hemi-liver for adult-to-adult LDLT [61]. This explains why LDLT has never exceeded more than 5 % of the total LT in the United States [6].

LDLT would likely shorten recipients' time to surgery, thereby preventing disease progression, which might occur while waiting for a deceased organ. One strong argument in favor of LDLT is that living liver donors, by reducing the number of recipients on the deceased donor waiting list, potentially advantage patients remaining on the waiting list. However patients with HCC beyond the accepted criteria for LT raise some ethical concerns. In order to analyze the appropriateness of LDLT, the concept of double equipoise could be used [62]. It describes the balance between the recipient's survival benefit with or without a live donor transplant and the probability of donor mortality risk [63–66]. This balance should be explicitly defined and agreed upon by all parties, including the recipient, donor, surgical team, and society.

Previous studies have reported conflicting results with respect to recurrence rates and overall survival after LDLT. Several studies comparing deceased donor liver transplantation (DDLT) and LDLT for HCC and 2 meta-analyses were published in the last decade [67–74]. Despite higher recurrence rates in three studies, the overall survival rates of LDLT for HCC compared to DDLT in all studies were not inferior. One could argue that this difference would eventually translate into a lower long-term survival in the LDLT groups. Given that LDLT is offered on a faster track than DDLT, it is conceivable that many LDLT recipients did not have sufficient waiting time to declare the biologic behavior of their HCC. In contrast, patients who await DDLT and who have a biologically aggressive HCC are likely to progress and then to dropout from the waiting list, leaving only patients with less aggressive HCC having access to DDLT. Of note, neither the waiting time, nor the type of graft (DDLT vs. LDLT) was identified as risk factors for HCC recurrence.

The first meta-analysis evaluated outcomes including patient survival, recurrence-free survival, and recurrence rates at defined time points in patients with HCC receiving a LDLT or a DDLT [73]. Seven studies with a total of 1310 patients were included in this study. For both LDLT and DDLT recipients, there was no significant difference in terms of overall survival rates (1 year, OR = 1.03, 95 % CI = 0.62–1.73; 3 years, OR = 1.07, 95 % CI = 0.77–1.48; and 5 years, OR = 0.64, 95 % CI = 0.33–1.24) and recurrence-free survival rates (1 year, OR = 0.86, 95 % CI = 0.54–1.38; 3 years, OR = 1.04, 95 % CI = 0.69–1.58; and 5 years, OR = 1.11, 95 % CI = 0.70–1.77). Moreover, there was also no significant difference regarding 1-, 3- or 5-year recurrence rates between LDLT and DDLT recipients (1 year, OR = 1.55, 95 % CI = 0.36–6.58; 3 years, OR = 2.57, 95 % CI = 0.53–12.41; and 5 years, OR = 1.21, 95 % CI = 0.44–3.32). A subgroup analysis revealed similar outcomes for patients with HCC meeting the MC. These findings demonstrate that for HCC patients (especially those within the MC), LDLT represents an acceptable option that does not compromise patient survival or increase HCC recurrence in comparison with DDLT.

The second meta-analysis included 16 studies, which were heterogeneous, non-randomized, and mostly retrospective [74]. The combined hazard ratio was 1.59 (95 %

CI = 1.02–2.49; I^2 = 50.07 %) for disease-free survival after LDLT vs. DDLT for HCC, and 0.97 (95 % CI = 0.73–1.27; I^2 = 5.68 %) for overall survival. This analysis provided evidence of lower disease-free survival after LDLT compared with DDLT for HCC. However, one contributing factor may be that HCC patients selected for LDLT may have worse tumor biology than DDLT. In the adult-to-adult living donor liver transplant study (A2ALL) there was a trend toward LDLT recipients having worse tumor characteristics but the type of graft (LDLT vs. DDLT) was a predictive factor of recurrence [75].

Then the question arises whether LDLT should be offered to HCC patients in whom tumor stage prevents the use of DDLT. Offering LDLT only to selected patients with advanced HCC cases is based on respect for the principles of donor autonomy and fairness. Since other listed patients are not adversely affected by this process, the required "acceptable" survival may be lower than the expected survival for other deceased donor indications. Such policy requires rigorous safeguards to ensure the pressure to treat recipients does not result in donor coercion, increased risk-taking by the donor surgical team, or donor depression after a poor LDLT outcome; and a minimum survival expectation needs to be established. On this difficult question, the jury of the 2010 international consensus conference on LT for HCC stated that there are currently no high-quality data to endorse or ban the use of different criteria for DDLT and LDLT for HCC [4]. Centers choosing to use different LT criteria for HCC in living donor liver transplants must carefully weigh respect for donor autonomy with the responsibility to protect the donor. Each center should explicitly state its policy regarding living donation for HCC patients with a poorer prognosis [76].

32.6 Is There a Place for Adjuvant Therapy After Liver Transplantation for HCC?

Efforts to decrease posttransplant liver recurrence rates and to further improve overall survival have included antitumoral adjuvant treatment after LT for HCC. Adjuvant therapy may achieve this goal through the elimination of undetectable micrometastases present at the time of the transplantation. However, because of possible adverse effects, the potential benefits of adjuvant therapy must be weighed against the risks. Furthermore it should be kept in mind that the use of frequent combined neoadjuvant or intraoperative therapies makes the assessment of the posttransplant adjuvant therapy more difficult. For instance, some patients may receive chemoembolization or a local treatment such as radiofrequency ablation before LT.

Taking into consideration these limitations, 8 non-randomized studies suggested a very modest benefit from adjuvant chemotherapy [77–84]. Four RCTs assessing adjuvant monotherapy or combined chemotherapy failed to demonstrate any benefit [85–88]. As listed in Table 32.1, two randomized studies using the single-agent doxorubicin during LT did not demonstrate any significant benefit [86, 87]. In the RCT from Li et al. [85], epirubicin was administrated in both groups and an adenovirus-mediated delivery of herpes simplex virus thymidine kinase therapy injected in the peritoneum in the experimental group was evaluated. Epirubicin alone did not show any survival benefit in advanced HCC patients. Interpretation of the results of the virus-mediated thymidine kinase therapy in such a patient population and with a very small sample size is very difficult. Similarly Folfox did not show any benefit on 3-year

Table 32.1 Randomized controlled trials assessing adjuvant therapies after liver transplantation for HCC

Authors (year)	Treatment	Treated patients/controls (n)	Follow-up (year)	Disease-free survival (treated patients/controls)	Overall survival (treated patients/controls)
Pokorny et al. (2005)[86]	Doxorubicin	34/28 (outside Milan)	5	43/53 % (NS)	38/40 % (NS)
Söderhahl et al. (2006)[87]	Doxorubicin	19/27 (outside Milan)	3	63/50 % (NS)	63/70 % (NS)
Li et al. (2007) [85]	Epirubicin in both groups + Thymidine kinase in peritoneum	23/22 (outside Milan)	3	43/9 % (p = 0.001)	69/20 % (p = 0.001)
Xu et al. (2007) [89]	Licartin	30/30 (outside Milan)	1	57/27 % (p = 0.017)	82/62 % (p = 0.001)
Zhang et al. (2011)[88]	FOLFOX	29/29 (outside Milan)	3	48/51 % (NS)	79/62 % (NS)

FOLFOX: Oxaliplatin+leucoverin+fluorouracil

disease-free or overall survival in HCC patients beyond MC [88]. Licartin, a 131I-radiolabeled murine monoclonal antibody that specifically binds to HCC cells expressing an HCC-specific molecule (HAb18G/CD147), was tested in a small placebo-controlled, randomized, double blind study in China [89]. Only a small number of HCC patients beyond MC were included and the 1-year follow-up was short. However, the benefit on recurrence rate and overall survival is encouraging but need to be confirmed at long-term. In summary, results from controlled studies are mixed, negative, inconclusive or requiring confirmation. As recommended by the last international consensus conference on LT for HCC, the current evidence does not justify the routine use of adjuvant antitumor therapy after LT for HCC outside of a controlled clinical trial [4].

Some hope has been placed in sorafenib, a multitargeted tyrosine-kinase inhibitor, which was shown to have an anti-tumoral effect in patients with advanced unresectable HCC [90]. A group from China designed a RCT of patients undergoing LT for HCC outside the MC and compared sorafenib with capecitabine to prevent recurrence after LT [91]. Thirty patients were randomized, with their follow-up ranging from 6 to 34 months. Treatment was started 1 month after LT and was discontinued 18 months later if no recurrence occurred or if there were severe adverse reactions. Although disease-free survival was longer in the sorafenib group, this was not significantly different compared with the capecitabine group. On the other hand, overall survival was significantly longer in the sorafenib group than in the capecitabine group. The authors concluded that for patients with HCC exceeding the MC, sorafenib may prolong survival with tolerable side effects. A case control study from Taiwan compared sorafenib as an adjuvant treatment or as a treatment for tumor recurrence in comparison with best supportive care [92]. Only 17 patients were considered, and all were beyond the MC. Patients in the adjuvant group received adjuvant sorafenib within the first 6 weeks after LT and, in the case of recurrence, until disease progression. Disease-free survival and overall survival were significantly longer for patients who received adjuvant sorafenib but overall survival was not significantly different between the recurrence and control groups. The authors suggested that adjuvant sorafenib could extend overall survival for HCC patients beyond the MC but that it is not effective as a palliative treatment after recurrence. These clinical data are supported by one experimental study in a transplant HCC rat model [93]. Sorafenib was administered after LT for 3 weeks, and it was highly effective in inhibiting cancer recurrence and metastasis without influencing the immune balance after LT for HCC. Because all these data are preliminary and suffered from small sample size and methodological biases, no conclusions on efficacy can be drawn.

Furthermore these positive results have been recently mitigated by the results of the STORM multicenter phase 3-trial, which tested sorafenib as adjuvant therapy after surgical resection, or local ablation of HCC [94]. Patients (n = 1114) were randomized either for sorafenib or placebo and the median follow-up for recurrence-free survival was 8.5 months (IQR 2.9–19.5) in the sorafenib group and 8.4 months (2.9–19.8) in the placebo group. There was no difference in median recurrence-free survival between the two groups (33.3 months in the sorafenib group vs. 33.7 months in the placebo group (HR = 0.940; 95 % CI 0.780–1.134, $p = 0.26$). These data indicate that sorafenib is not an effective treatment in the adjuvant setting for hepatocellular carcinoma following resection or ablation. Similarly, there was no significant treatment effect on the secondary endpoints of time to recurrence and overall survival.

32.7 What Is the Impact of Immunosuppression on HCC Recurrence After Liver Transplantation?

Despite the careful selection of HCC patients for LT, 10–20 % of liver transplant recipients who have HCC in the native liver develop tumor recurrence after transplantation-mainly within the first 2 years [5, 95]. In this setting, the main concern comes from immunosuppression therapy, which inhibits the tumor suppressive properties of the immune system and therefore, may increase the likelihood of HCC recurrence after LT. Indirect evidence of a favoring effect of immunosuppressant on tumor genesis comes from the observation that the incidence of malignancies is significantly higher in organ recipients than in the general population [96]. Besides the calcineurin inhibitors (CNIs), namely cyclosporine and tacrolimus, a newer category of immunosuppressant drugs called m-TOR (mammalian target of rapamycin) inhibitors raised a high degree of interest. Indeed these drugs are associated with strong immunosuppressant activity, due to the blocking of IL-2 stimulation of lymphocyte proliferation, and have a potential anti-cancer effect, which has been demonstrated in the experimental setting. The anti-cancer effect is mainly related to the impairment of vascular endothelial growth factor (VEGF) production and the blockage of VEGF-induced vascular endothelial cell stimulation [97, 98].

Experimental data provided good evidence that CNIs promote cellular growth of malignant cells by enhancing cancer cell invasions and by inhibiting DNA repair [99, 100]. On the other hand, mTOR inhibitors like sirolimus (SRL) inhibit hepatoma cell proliferation in vitro and down-regulates vascular endothelial growth factor

Table 32.2 Studies investigating on HCC recurrence after liver transplantation in patients receiving sirolimus as immunosuppression

Authors	Year	Type of study	Patients (n)	Outcomes
Zhou et al. [107]	2008	Retrospective cohort	73	6-month recurrence rate: 4 % vs. 20 %[a]
Zimmerman et al. [108]	2008	Retrospective cohort	97	5-year DFS: 79 % vs. 54 %[a]
Chinnakotla et al. [103]	2009	Case control	227	5-year DFS: 80 % vs. 59 %[a]
Toso et al. [104]	2007	Retrospective cohort	70	Recurrence 6 % for Milan vs. 17 % over Milan
Vivarelli et al. [106]	2010	Matched cohort	62	3-year DFS: 86 % vs. 56 %[a]
Toso et al. [105]	2010	Retrospective cohort	2491	Patient survival: hazard ration = 0.53

[a]Patients treated with sirolimus versus calineurin inhibitors; *DFS* Disease-free survival

expression. In animal models, rats receiving SRL had significantly longer survival and developed smaller tumors, fewer extrahepatic metastases compared to controls [101, 102].

In the last decade, clinical studies investigated whether mTOR inhibitors may affect the posttransplant recurrence rate of HCC [103–108]. As reported in Table 32.2, these studies showed significant benefit on HCC recurrence rates after LT in patients receiving SRL as immunosuppressant. However, because none of these studies were randomized, there is a significant potential for selection, treatment or reporting bias towards more positive findings of SRL.

In 2011, a first meta-analysis was conducted to determine if using SLR based regimens as immunosuppression after LT for HCC improves survival and recurrence [109]. Five studies with a total of 2950 patients were included [103, 105–108]. The pooled results showed that in comparison with SRL-free regimens, SRL-based regimens decreased tumor recurrence (OR = 042, 95 % CI = 0.21–0.83) and improved 5-year overall survival (OR = 2.47, 95 % CI = 01.72–3.55). However, as stated by the authors themselves, since none of the included studies performed a statistical analysis of the etiology of death, this meta-analysis could not determine whether the survival improvement was due to SRL itself or the CNI reduction in the protocol considering the nephrotoxicity and other side effects of CNIs. Other limitations of this meta-analysis are the lack of randomized controlled trials resulting on a potential selection bias, the lack of subgroup analyses based on potential confounding factors, and the fact that the analysis of each endpoints were based on only 2 or 3 included studies because of missing data. In 2013, an updated meta-analysis [110] included 5 studies with a total of 474 patients who underwent LT for HCC [103, 104, 106, 108, 111]. The tumor recurrence rate was lower in SRL group (4.9–12.9 %) in comparison with CNIs (17.3–38.7 %). The 1-, 3- and 5-year recurrence-free survival was 93–96, 82–86 and 79–80 % for SRL group, which was higher in comparison with the CNIs (70–78, 64–65 and 54–60 %, respectively). Similarly, 1-, 3- and 5-year overall survival was better in SRL group (94–95, 85 and 80 %) in comparison with CNIs (79–83, 66 and 59–62 %) respectively. This meta-analysis demonstrated lower recurrence (OR = 0.30, 95 % CI = 0.16–0.55, $p < 0.001$), lower recurrence-related mortality (OR = 0.29, 95 % CI = 0.12–0.70, $p = 0.005$) and lower overall mortality (OR = 0.35, 95 % CI = 0.20–0.61, $p < 0.001$) in the SRL group. More recently, a systematic review compared Everolimus (another mTOR inhibitor) and SRL with CNI use on post-LT recurrence of HCC [112]. It included 42 studies with 3.666 HCC LT recipients. CNI use was associated with higher rates of HCC recurrence compared to mTORs (13.8 vs. 8 %, $p < 0.001$), although patients treated with CNIs had lower rates of microvascular invasion and a higher proportion of HCC within MC. A subgroup analysis demonstrated that although patients taking Everolimus had shorter follow-up data, overall HCC recurrence post-LT was less frequently observed compared to SRL use (4.1 % vs. 10.5 %, $p = 0.02$).

Although retrospective and uncontrolled studies favor the use of mTOR inhibitors in LT for HCC patients, confirmatory data from a hypothesis-driven RCT are still missing. Up to now, no recommendation can be made for choosing any type or dose of immunosuppressant to influence the incidence or the prognosis of HCC recurrence after LT. The Silver 05 multicenter RCT studying the potential benefits of SRL use in this setting will definitely help to answer this question and is estimated to be completed in 2018 [113].

32.8 Conclusion

In the past 20 years LT for HCC rapidly developed as one of the most successful treatments in oncology. Undoubtedly an increasing number of HCC patients will have access to LT thanks to the acceptance of extended selection criteria, earlier tumor detection, control of tumor load while patients wait for a graft, use of living donors, tailored immunosuppression and adjuvant therapies. Some efforts should be made to better understand the tumor biology and prognostic factors in order to optimally select HCC patients who can

benefit from LT. In the context of organ shortage, the success of LT for HCC is not without ethical problems with respect to end stage liver disease patients and should prompt us to increase the pool of organs.

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Medical Therapy of HCC

33

Brian I. Carr, Srikanth Nagalla, and Ravit Geva

Contents

33.1 Principles

33.1.1 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 33.1). The male:female ratio was 2.5:1 with 72 % of our patients being Caucasian. Interestingly, 24 % of

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Table 33.1 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)
Other (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 (year)	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 %	

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. 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 proceeding 12 months of the diagnosis (Chap. 24). This appears to be a sensitive but nonspecific 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 33.1, 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 33.2).

Table 33.2 Treatment options for hepatocellular carcinoma

Potentially curative options
1. Liver resection
2. Liver transplantation
3. Ablative therapies: cytoreductive therapies
Palliative resection
Cryosurgery
Microwave ablation
Ethanol injection
Acetic acid injection
Radiofrequency ablation
4. Transcatheter hepatic artery treatments
Transarterial chemotherapy
Transarterial embolization
Transarterial chemoembolization (TACE)
Transarterial radiotherapy
^{90}Y microspheres (Sirspheres or Theraspheres)
^{131}I Lipiodol, ^{66}Ho, ^{188}Re
Gene therapies
5. External beam conformal radiation
6. Systemic therapies
Chemotherapy Molecularly targeted therapies
Immunotherapy
Hormonal therapy
Growth factor or antibody control of cell cycle
7. Supportive (Palliative) care

33.1.2 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), 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. 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.

33.1.3 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 33.1, 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 two-fold. 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.

33.1.4 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. 33.1) or on hepatic angiography (Figs. 33.2, 33.3, 33.4 and 33.5). 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 supply to HCC typically arises from hepatic arteries, whereas the delivery of 90 % of the oxygenated blood to the underlying nontumorous liver is mainly from the portal vein. This provides a partial basis for intrahepatic chemoembolization or intrahepatic chemotherapy, which permits a relatively selective delivery of chemotherapy to the tumors in the liver via the tumor neovasculature that typically grows in response to the presence of an HCC. The other reason is that vascular slowing leads to an increase in the hepatic dwell time of infused chemotherapy.

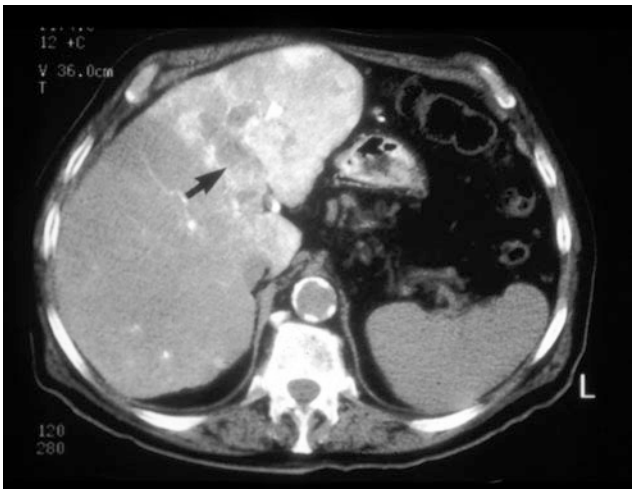

Fig. 33.1 CAT scan showing a vascular HCC and portal vein thrombosis (PVT)—*arrow*

33.1.5 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. 33.1)

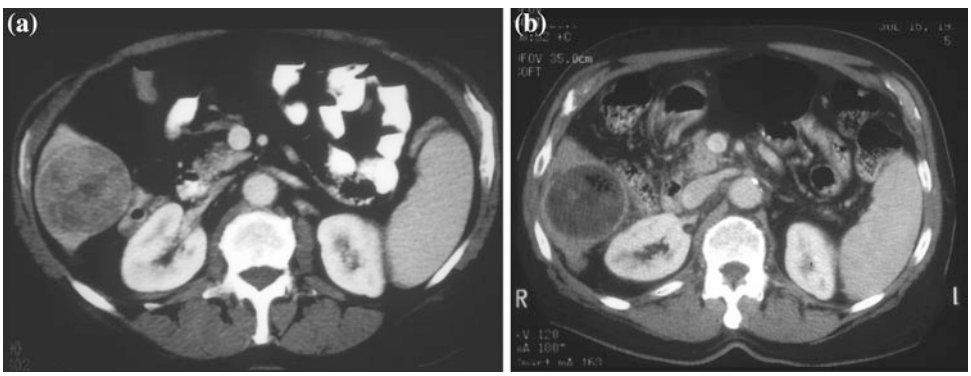

Fig. 33.2 CAT scans showing a vascular response to TACE (chemoembolization) without size response (**a** pre-therapy; **b** post-therapy)

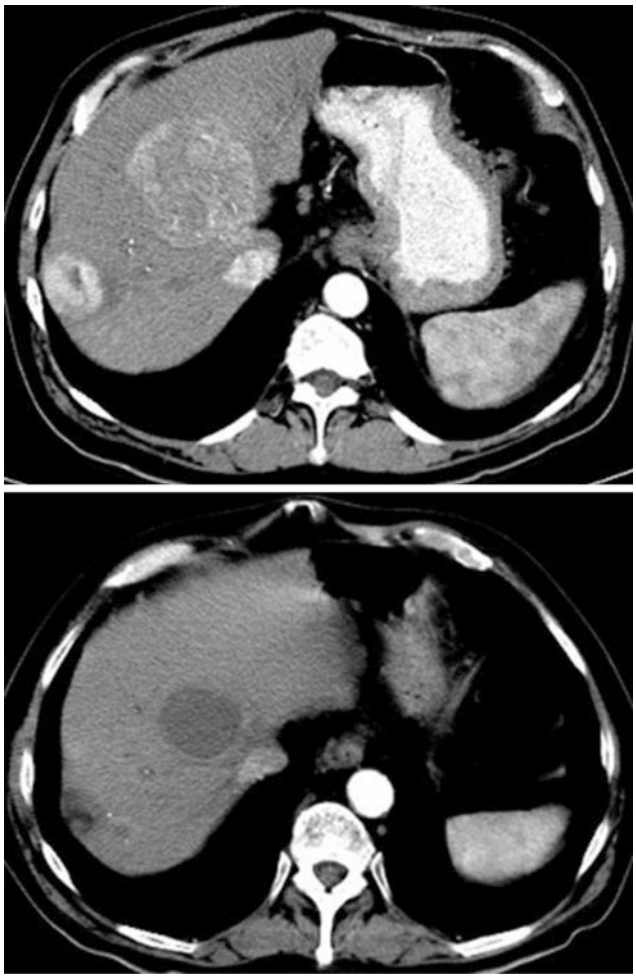

Fig. 33.3 CAT scan showing both size responses (tumor shrinkage) and vascular responses-change from hypervascular to hypo vascular lesions (*upper* pre-therapy; *lower* post-therapy)

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 contra-indication 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 chemoocclusion 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 subocclusion but never complete embolization of the treated hepatic artery.

33.1.6 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

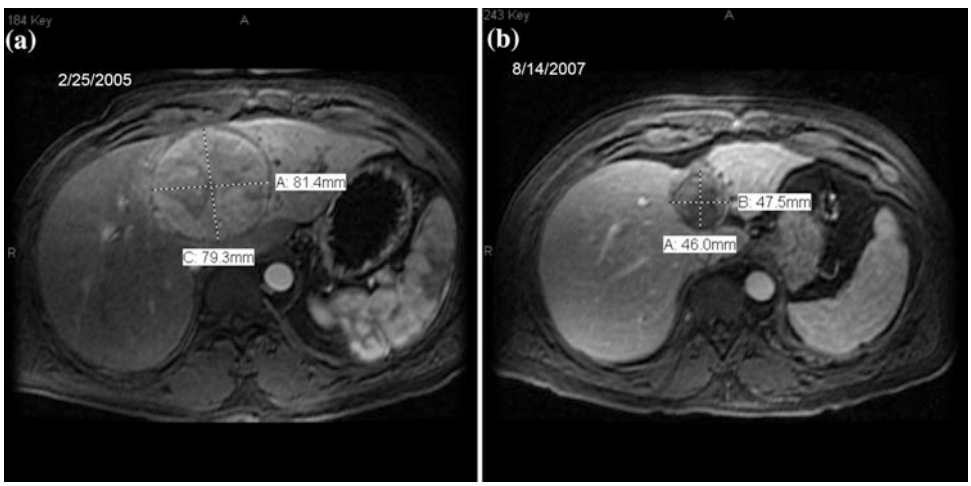

Fig. 33.4 CAT scans showing partial response (PR) in tumor size and vascular response of the same mass (**a** pre-therapy; **b** post-therapy)

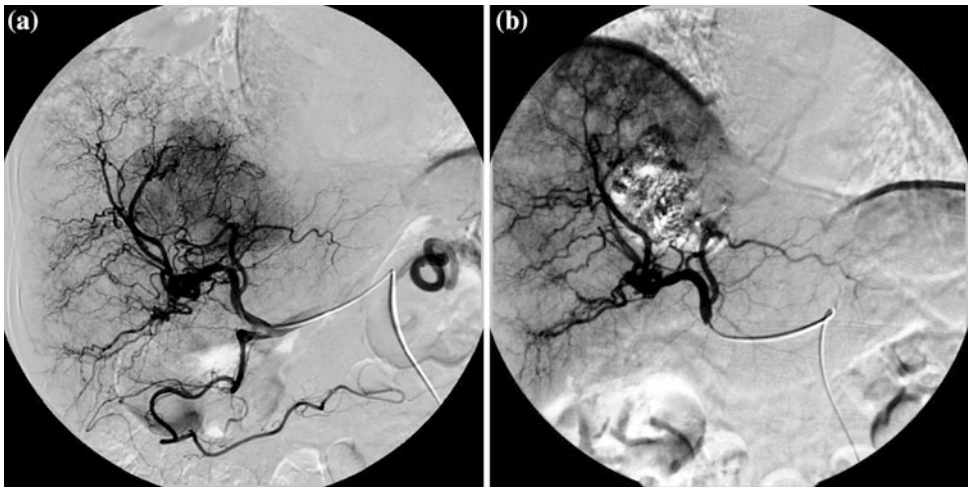

Fig. 33.5 Hepatic angiogram showing change in tumor vascularity postchemoembolization (TACE) (**a** pre-therapy; **b** post-therapy)

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 II and phase III chemotherapy drugs have shown responses to single drugs in less than 20 % of the patients and have no beneficial effect on survival (Table 33.3).

33.1.7 Hepatic Artery Chemotherapy

However, when the same drugs are given by the hepatic artery route, they have been found to result 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 33.4 and 33.7). 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 33.5, 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 33.6), using cisplatin [8] or doxorubicin [9], respectively.

33.2 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:

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, leukopenia's and thrombocytopenia's consequent to splenomegaly is 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 3000/L, or platelet count greater than 40,000/ml. The recent introduction of granulocyte colony stimulating factors (CSFs), such as pegfilgastrim (Neulasta) into clinical practice, means that the WBC can be restored to safe levels by the oncologist at will.
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.
3. The cirrhotic liver has decreased xenobiotic metabolizing capacity
   The decreased metabolic capacity and 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

Table 33.3 Selected recent studies of chemotherapy

		Partial response rate
Investigations	Drug	(%)
Systemic chemotherapy		
Sciarrino et al. 1985 [78]	Doxorubicin	0
Chlebowski et al. 1984 [79]	Doxorubicin	11
Ihde et al. 1977 [80]	Doxorubicin	15
Falkson et al. 1984 [81]	Doxorubicin, 5-fluorouracil, methyl-CCNU	19
Falkson et al. 1984 [82]	Neocarzinostatin	8
Ravry et al. 1984 [83]	Doxorubicin, bleomycin	16
Cavalli et al. 1981 [84]	VP-16	13
Melia et al. 1983 [85]	VP-16	18
Melia et al. 1981 [86]	Cisplatin	1
Ravry et al. 1986 [87]	Cisplatin	0
Falkson et al. 1987 [88]	Cisplatin	17
Falkson et al. 1987 [88]	Mitoxantrone	8
Colleoni et al. 1993 [89]	Mitoxantrone	23
Chao et al. 1998 [90]	Paclitaxel	0
Patt et al. 2003 [91]	5-FU + IFN	18
Patt et al. 1999 [92]	5-FU + IFN + Cisplatin + Doxorubicin	20
Bobbio-Pallayicini et al. 1997 [93]	Epirubicin + VP-16	39
Okada et al. 1999 [94]	Cisplatin, mitoxantrone + 5-FU	33
Guan et al. 2003 [95]	Gemcitabine	2
Taïeb et al. 2003 [96]	Gemcitabine, oxaliplatin	19
Lee et al. 2004 [97]	Doxorubicin, cisplatin	19
Ikeda et al. 2005 [98]	5-fluorouracil, mitoxantrone, cisplatin	27
Zhu et al. 2005 [99]		0
Zhu et al. 2006 [100]	Epirubicin, thalidomide	20
Kim et al. 2006 [101]	Gemcitabine, oxaliplatin, bevacizumab	17
Park et al. 2006 [102]		24
Louafi et al. 2007 [61]	Epirubicin, cisplatin, UFT, leucovorin	18
Li et al. 2007 [103]		2
Uhm et al. 2008 [104]	Doxorubicin, cisplatin, capecitabine	16
Asnacios et al. 2008 [105]	Gemcitabine, oxaliplatin	20
	Gemcitabine, oxaliplatin	
	Oxaliplatin, doxorubicin	
	Gemcitabine, oxaliplatin, cetuximab	
Yeo et al. [21]	PIAF, platinum, interferon, adriamycin, fluorouracil	21
Reviews [106–113]		

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.

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.

33.3 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.

33.3.1 Commonly Used Drugs

Chemotherapeutic agents that have been commonly used in many centers include cisplatin or cisplatin (Platinol), doxorubicin (Adriamycin), 5-FUdR, mitomycin C, in addition to the much lower experience with neocarzinostatin (SMANCS), and, gemcitabine (Gemzar) (Table 33.5). They have been used as single agents and in combinations, with (usually) or without some form of embolizing agent to produce chemoembolization or chemoocclusion. However, there is little data or agreement of the number of agents to be infused, with one is the same as more than one agent, and which agent (s) is superior. Until this can be resolved, the evidence favors use of either cisplatin [8] or doxorubicin [9] (Table 33.6). 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 [10, 11] (Table 33.7).

Table 33.4 Intrahepatic artery chemotherapy for hepatocellular carcinoma

Investigation	Agents	Response rate (%)
Sasaki et al. 1987 [114]	Platinum—gelatin sponge	65
Kasugai et al. 1989 [115]	Platinum—ethiodized oil	38
Ohnishi et al. 1984 [116]	MMC—microcapsules	32
Lin et al. 1988 [117]	5-FU—Ivalon	32
Fujimoto et al. 1985 [118]	5-FU/MMC—starch	68
Audisio et al. 1990 [119]	MMC + microcapsules	43
Kobayashi et al. 1986 [120]	Doxorubicin + ethiodized oil	42
Kanematsu et al. 1989 [121]	Doxurubicin + ethiodized oil	47
Shibata et al. 1989 [122]	Platinum + ethiodized oil	47
Konno et al. 1983 [123]	SMANCS + ethiodized oil	90
Pelletier et al. 1990 [124]	Doxorubicin + gelatin sponge	17
Carr et al. 1991 [125]	Doxorubicin/cisplatin	50
Venook et al. 1990 [126]	Doxorubicin/cisplatin/MMC + gelatin sponge	24
Ohnishi et al. 1987 [127]	MMC + microcapsules	28
Ohnishi et al. 1987 [127]	MMC + gelatin sponge + microcapsules	57
Beppu et al. 1991 [128]	Cisplatin + ethiodized oil + aclarubicin microspheres	50
Trinchet et al. 1995 [129]	Cisplatin + ethiodized oil versus 0	16
Chang et al. 1994 [130]	Cisplatin + gelfoam + ethiodized oil versus gelfoam + ethiodized oil	68[a] 67[a]
Stuart et al. 1993 [131]	Doxorubicin, ethiodized oil + Gelfoam	43
Bruix et al. 1994 [132]	Gelfoam, no chemotherapy	81
Carr et al. 1997 [133]	Doxorubicin, cisplatin + Spherex	63
Carr et al. 1993 [134]	Doxorubicin, cisplatin + ethiodized oil versus doxorubicin + cisplatin	57 47
Carr et al. 2002 [135]	Cisplatin	58
Ngan et al. 1993 [136]	Cisplatin, ethiodized oil, gelfoam	41
Yamamoto et al. 1993 [137]	IL-2	
Kawai et al. 1994 [138]	Epirubicin + gelfoam versus doxorubicin + gelfoam	[a]
Yoshimi et al. 1992 [139]	Resection versus TAE	[a]
Epstein et al. 1991 [140], [141]	Cisplatin + hepatic radiation	48
Rougier et al. 1993 [141]	Doxorubicin + gelfoam	41
Onohara et al. 1988 [142]	Cisplatin	55
Kajanti et al. 1986 [143]	Cisplatin	40
Nagasue et al. 1986 [144]	Epirubicin	15
Carr et al. 1996 [10] Lin et al. 2004 [145] Jang et al. 2004 [146] Carr 2006 [147]	Cisplatin dose escalation Cisplatin, mitomycin C, 5-FU and leucovorin 5-FU and cisplatin Gemcitabine	50 28 29

5-FU 5-fluorouracil; *MMC* Mitomycin C; *SMANCS* Styrene maleic acid conjugates of neocarzinostatin and mitomycin C; *IFN* Interferon
[a]Similar survival

33.3.2 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 microns. 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 [12]. 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 effect of Lipiodol to chemotherapy in terms of tumor response [13]. 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 [14]. 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.

33.3.3 Protocol for Chemoocclusion Therapy of HCC

Our largest experience has been with cisplatin. This is based upon the fact that it has moderate tumor shrinking ability and has minimal myelosuppressive activity compared with most

Table 33.5 Some randomized clinical trials involving transhepatic artery chemoembolization versus other chemotherapy for HCC

Author	Year	Agents 1	Agents 2	Effects on survival
Kawai [148]	1992	Doxorubicin + embo	Embo	None
Kawai [149]	1997	Epirubicin + embo	Doxorubicin + embo	None
Watanabe [150]	1994	Epirubicin + embo	Doxorubicin + embo	None
Chang [130]	1994	Cisplatin + embo	Embo	None
Hatanaka [151]	1995	Cisplatin, doxorubicin + Embo	Same + lipiodol	None
Uchino [152]	1993	Cisplatin, doxorubicin + oral FU	Same + tamoxifen	None
Madden [153]	1993	Cisplatin + ADMOS	5-epi-doxorubicin	None
Chung [154]	2000	Cisplatin + 1FN	Cisplatin	None
Lin [117]	1988	Embo	Embo + IV FU	None
Yoshikawa [155]	1994	Epirubicin + lipiodol	Epirubicin	None
Kajanti [156]	1992	Epirubicin + FU	IV Epirubicin + FU	None
Tzoracoleftherakis [157]	1999	Doxorubicin	IV Doxorubicin	None
Bhattachariya [158]	1995	Epirubicin + lipiodol	^{131}I-Lipiodol	None

Table 33.6 Randomized clinical trials involving transhepatic arterial chemoembolization (TACE) chemotherapy versus no treatment controls

Author	Year	Agents	Effects on survival
1. Pelletier [124]	1990	Doxorubicin + gelfoam	None
2. Trinchet [129]	1995	Cisplatin + gelfoam	None
3. Bruix [159]	1998	Coils and gelfoam	None
4. Pelletier [160]	1998	Cisplatin + lipiodol	None
5. Lo [8]	2002	Cisplatin + lipiodol	Yes
6. Llovet [9]	2002	Doxorubicin + lipiodol	Yes
7. Reviews [14, 108, 109, 161]			

Table 33.7 Effects of hepatic arterial cisplatin dose intensity [10, 11]

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 (mo) ± SE:		
	Cisplatin alone	Cisplatin + Gelfoam
Responders	29.0 ± 3.5	25.5 ± 1.7
Nonresponders	11.1 ± 1.5	15.6 ± 3.1
	$p < 0.0001$	$p < 0.003$
C. Effect of treatment type on median survival (mo) ± SE:		
	Cisplatin alone	Cisplatin + Gelfoam
	19.53 ± 6.3	30.73 ± 0
	$p < 0.137$	
D. Effect of dose density on median survival (mo) ± SE:		
	Cisplatin alone	Cisplatin +Gelfoam
Dose = or < 125 mg/m^2/mo	9.9 ± 1.66	16.4 ± 2.8
Dose = > 125 mg/m^2/mo	19.5 ± 7.2	30.7 ± 0
	$p < 0.07$	$p < 0.69$

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 2 or 3 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. 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 D5$^1/_2$ normal saline or just 1/2 normal saline with 20 mg KCl per 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 antiemetics 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 postchemotherapy 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 2 or 3 administrations after the vascular occlusion. The pain of the postembolization syndrome is likely due in part to arterial spasm. Lab work is rechecked 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 Chap. 21.

The chemotherapy (Trans Arterial Chemo Embolization, 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 posttreatment. 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.

33.4 Safety Considerations of Hepatic Artery Chemoocclusion

33.4.1 Unilobar Treatments Are Typically Given at Any Single Therapy Session

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 chemoocclusion 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.

33.4.2 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.

33.4.3 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 33.7) through 150–175 mg/m^2, although few patients can tolerate the last. A completely normal blood count and no change in liver function tests is used as the basis 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

2000 × 10^3/Ll or nadir platelet count above 40,000 × 10^9/Ll 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 2 or 3 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 regrowth.

33.5 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 upon prolonged survival, greater than 24 months, poor survival, less than 4 months, or intermediate between these two (Tables 33.8, 33.9, and 33.10). 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 33.8). 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 33.9). 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

Table 33.8 Cisplatin hepatic artery chemoembolization: prognostic factors for survival (n = 155)

Patient characteristics (% pts)			
Patient survival			
	>24 mo	4–24 mo	<4 mo
	n = 49	n = 80	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 33.9 Cisplatin hepatic artery chemoembolization: prognostic factors for survival (n = 155)

Tumor characteristics (% pts)			
Patient survival			
	>24 mo	6–24 mo	<6 mo
	n = 49	n = 80	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 K ng/ml	12	30	46
Response to chemotherapy			
Chemo responses (PR)	84	69	8
Tumor stability	16	25	4

Table 33.10 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

by tumor shrinkage or decreased tumor vascularity on a triple-phase helical CT scan was strongly associated with the prolonged survival group (Table 33.9). Examples of this are shown in the CT scans and angiograms in Figs. 33.1, 33.2, 33.3, 33.4, and 33.5. 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. 33.3 and 33.4), as well as a decrease in tumor vascularity [15, 16] (Fig. 33.2). 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 33.10). 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 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 33.10). 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 33.10). The new era of kinase inhibitors and antiangiogenic agents, 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, as well as because the newer agents such as sorafenib enhance survival with minimal associated scan tumor responses [17]. 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.

33.5.1 TACE Using Drug Eluting Beads

Drug eluting beads (DEB) 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. Majority of the studies have been done only with doxorubicin-containing DEB with an occasional study using epirubicin [18]. The results seem to be promising with response rates anywhere from 50 to 81 %, similar to TACE, and with a good safety profile. The PRECISION V trial demonstrated improved outcomes (higher rates of complete response (CR), objective response and disease control at 6 months) for TACE with doxorubicin DEB compared to conventional TACE. The above-mentioned outcomes were achieved with less liver toxicity and doxorubicin-associated side effects [19]. A recent meta-analysis by Huang et al. demonstrated that DEB-TACE had a better objective tumor response rate than conventional TACE without any overall survival benefit. There was no significant difference in the toxicity profile between the two arms [20]. Large randomized control trials in the future will be needed to give us definitive answers regarding the efficacy of these agents.

33.6 Systemic Therapy

33.6.1 Chemotherapies

A huge number of randomized and nonrandomized studies have been performed with various single agents and some combinations of chemotherapeutic agents (Table 33.3). In Table 33.3 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 [21] are associated with enhanced toxicity but without a survival benefit. 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 Chap. 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.

33.6.2 Sorafenib

Two phase III randomized trials demonstrated the benefit of sorafenib in advanced HCC. The SHARP trial conducted in North America and Europe demonstrated a median overall survival benefit of 10.7 months for sorafenib compared to 7.9 months for placebo [17]. The time to progression was 5.5 months with sorafenib compared to 2.8 months with the placebo. A similar study was conducted in Asia and the median overall survival for patients on sorafenib was 6.5 months compared to 4.2 months with a placebo [22]. Some of the important side effects include diarrhea, rash/desquamation, hand-foot skin reaction, hypertension, and hypoalbuminemia. The GIDEON phase IV trial evaluated the safety of sorafenib in the real-world setting [23]. An interim analysis in 2011 demonstrated that Child Pugh A patients had a median survival of 10.3 months compared to 4.8 months for Child Pugh B patients. The STORM trial evaluated the benefit of sorafenib in the adjuvant setting. The findings were reported at ASCO 2014

by Bruix et al. There was no difference in the recurrence-free survival, time to recurrence, or overall survival between sorafenib and placebo in this trial.

33.7 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 upon 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 33.11). Despite initial reports of responses to Tamoxifen, subsequent controlled randomized trials have essentially shown no survival benefit for Tamoxifen, LHRH antagonists such as Luprolide or Flutamide, or Megesterol. A similar large number of studies have investigated the effects of interferons, both because they have an antiangiogenic action as well as an antihepatitis activity. Although there are conflicting reports of benefit or no benefit to tumor shrinkage or survival, the consensus is there is 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 [24–26]. 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 supratherapeutic doses. However, two recent randomized trials from Japan show that oral K vitamins can decrease postresection recurrences, as well as decrease the incidence of HCC in HCV carriers [27–29].

Table 33.11 Various recent medical treatments evaluated for unresectable HCC

A. Systemic
Tamoxifen [162]
LHRH agonists [163, 164]
Interferon [165]
Sandostatin [166]
Megestrol [167, 168]
Vitamin K [25, 169]
Thalidomide [170]
EGFR antibody [171]
Arsenic trioxide [172]
IL-2 [137]
Antiangiogenesis strategies [173] Immunotherapy [174]
B. Hepatic arterial
^{131}I-Lipiodol [175]
^{131}I-Ferritin [176]
^{90}Yttrium microspheres [30]

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 33.11).

Although HCC is thought to be in general a radio-resistant tumor, there is some evidence of antitumor activity with radioactively administered agents delivered into the hepatic artery, including ^{131}I-Lipiodol, ^{188}Re-Lipiodol, ^{166}Ho, and ^{32}P. 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 [30]. 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 33.6 shows a CT scan demonstrating a CR with this therapy and Fig. 33.7 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 [31]. 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 is shown in Fig. 33.8. A randomized comparison of ^{90}Yttrium (Therasphere or Sirspheres) with intrahepatic chemotherapy will be needed to determine whether one treatment or the other is associated with prolonged survival and increased quality of life.

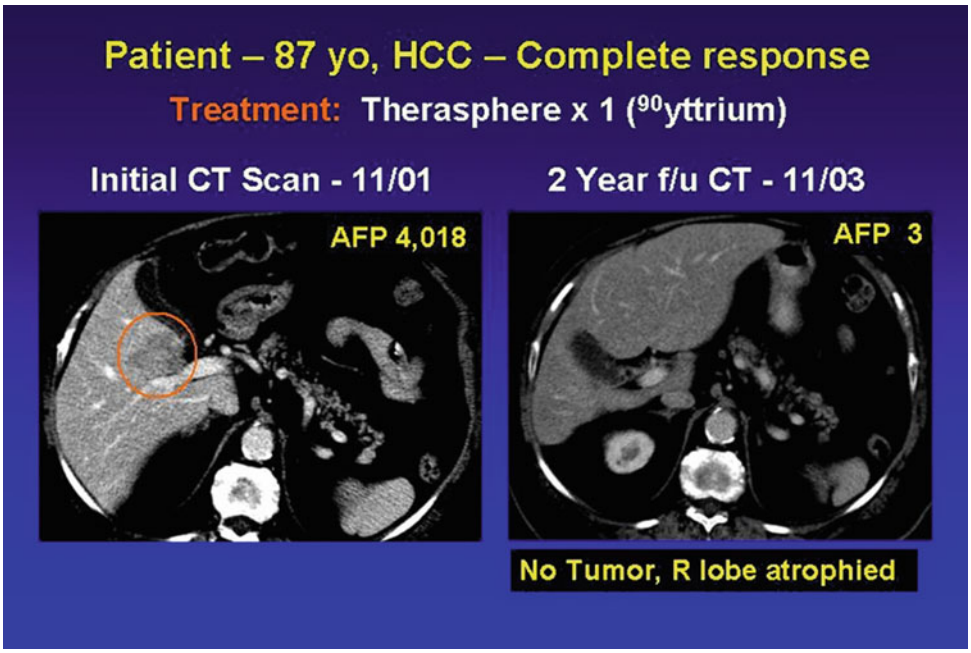

Fig. 33.6 CAT scan showing complete tumor disappearance or complete response (CR) post-Theraphere ^{90}Yttrium therapy, together with right hepatic lobe atrophy (*left* pre-therapy; *right* post-therapy)

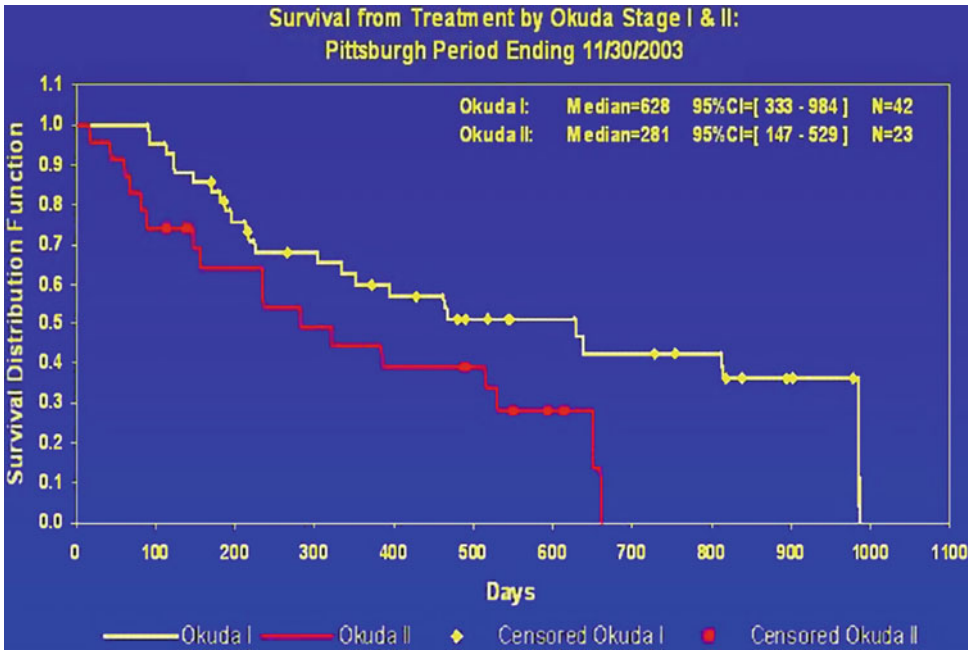

Fig. 33.7 Survival analysis after Theraphere ^{90}Yttrium therapies, according to Okuda stage I (*upper curve* Median 628 days) or stage II (*lower curve* Median 281 days)

33.8 Treatments for Metastatic HCC

33.8.1 Immunotherapy

Different forms of immunotherapy have been tested in HCC with few and conflicting results.

Interferon alfa—an immunomodulatory cytokine/its anti tumoral activity have been demonstrated in a subset of patients mainly with metatstatic melanoma. Small series describing mainly good tolerability are available. Three small randomized trials tested its efficacy in metastatic HCC. In one of the trials IM daily recombinant alpha 2 interferon (rIFN) was randomized versus ADR in 75 Chinese patients. rIFN showed higher tumor regression with less toxicity [32]. A second promising trial compared INFa versus best supportive care (BSC) in 71 Chinese patients demonstrating an improved survival with relatively little toxicity [33]. However, a similarly designed European trial investigated 58

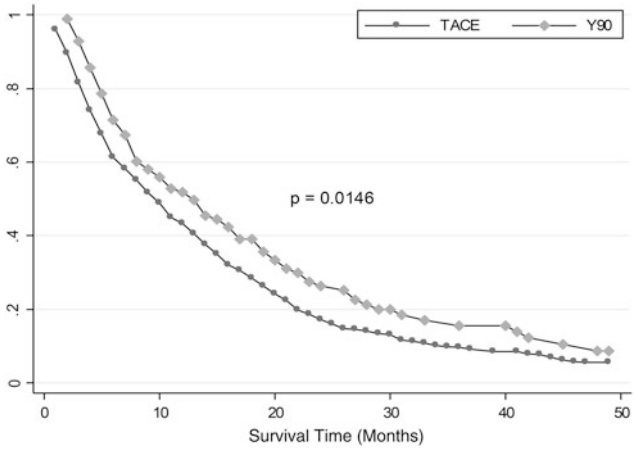

Fig. 33.8 Survival curves for 2 consecutive patient cohorts treated with either Theraphere ^{90}Yttrium therapy (*upper curve*) or chemoembolization (TACE, *lower curve*). Therasphere-treated patients had longer survival, $p = 0.0146$

patients randomized to INFa given three times a week versus BSC failed to show any advantage and side effects lead to treatment discontinuation in 13 out of 23 patients [34].

33.8.2 Anti-programmed Death-1 (PD-1)

In 2011, tumor immune evasion mechanisms were added to our understanding of the multistep development of human tumors in the "hallmark of Cancer" pivotal paper [35]. PD-1 is a cell surface receptor that plays an important role in down regulating the immune system. PD-1 binds to two ligands, PD-L1 and PD-L2. Anti-PD1/PDL1 antibodies are currently extensively researched in solid tumors and have demonstrated significant efficacy in melanoma, lung cancer, and renal cell carcinoma. In HCC, several evasive mechanisms utilizing PD-1 have been described including upregulation of PD-1 expression on effector-phase CD8+ T cells and PD-L1 expression on Kupffer cells [36].

Recently, a phase II study of nivolumab, a fully human IgG4 monoclonal anti-PD1 was presented at ASCO 2015 [37]. Forty-one patients Child-Pugh $\leq$ B7 who and progressived, were intolerant or refused sorafenib were enrolled, 39 were evaluable. Two CR (5 %) were demonstrated with an additional 18 % partial response. Duration of response reached 14–17+ months for CR patients. Overall survival rate at 6 months was 72 %. Anti-PD1s are considered well tolerated and in this study no maximum tolerated dose was defined in any cohort. The role of anti-PD1 agents is still premature to determine and phase III trials are now ongoing.

33.8.3 Multikinase Inhibitors

33.8.3.1 Sorafenib/Nexavar

This has become the standard therapy for extra-hepatic HCC metastases and the only FDA-approved oral therapy for HCC. It is prominently discussed in section **F(2)** above and in Chap. 36: Multikinase Inhibitors for HCC.

33.8.3.2 Antivascular Endothelial Growth Factor

Most of the available data are with trials testing Bevacizumab which is an antiangiogenic, anti-VEGF monoclonal antibody. Its efficacy has been demonstrated in several solid tumors but was proved only when combined with chemotherapy. In metastatic HCC, some single agent activity was suggested [38, 39]. Bevacizumab was tested with several combinations. Bevacizumab and Erlotinib, an EGFR TKI, showed promising synergism in 2009 in a group of 59 patients when 25 % confirmed RR was seen [40]. An Asian trial with 51 patients showed a 16-week PFS 35.3 % [41], both trials with good tolerability. However, the combination failed to show any efficacy in sorafenib refractory patients in a trial that was closed after 10 patients for futility [42]. Bevacizumab was tested with another antiangiogenic agent temsirolimus in 28 patients with 19 % RR [43].

Combination of Bevacizumab with chemotherapy was also tested. Efficacy of Bevacizumab with single agent capecitabine in 45 patients was 9 % with disease control rate of 52 % [44]. The combination with gemcitabine and oxaliplatin (GEMOX) was tested on 33 patients with RR of 20 % and an additional 27 % of patients with stable disease. Toxicity was predictable for combination chemotherapy 181. The antiangiogenic effects were confirmed when this combination effect was examined with perfusion CT and a significant decrease tumor perfusion parameters were seen in responding patients [45]. No confirmation randomized trials are available for any of the regimens.

Another antiangiogenic monoclonal antibody rested is Ramucirumab which binds to VEGFR2. A phase II trial of 42 in naïve patients a 9.5 % was demonstrated [46]. Efficacy of ramucirumab was tested following failure of sorafenib versus placebo in a large phase III trial (the REACH trial) incorporating 565 patients. The trial failed to show a statistically significant advantage in this large set of patients. However, some survival advantage was suggested in a subset of patients with AFP > 400 ng/mL and a phase III testing this specific subset is ongoing [47].

33.8.3.3 Antiepidermal Growth Factor

Some data is available for the role of anti-EGFR TKI in metastatic HCC. This specified earlier on and is discussed further in

the chapter devoted to TKIs. Cetuximab, an anti-EGFR monoclonal antibody was tested in combination with GEMOX. In 45 naïve patients, a confirmed RR was 20 % and disease stabilization was achieved in 40 % of patients [48].

33.9 Systemic Chemotherapy

33.9.1 Single Agent Chemotherapy

Doxorubicin (ADR) is the most extensively studied single agent chemotherapy. Results have ranged from complete futility to almost 80 % response. Only one sufficiently large phase III trial is available from an Italian group, which randomized 445 patients to ADR or nolatrexed. Survival with ADR reached 32.3 weeks and only 22.3 weeks with nolatrexed ($P = 0.0068$) [49].

Toxicity profile in most series was not favorable with high rates of neutropenia, septicemia, and cardiotoxicity questioning the worthiness of ADR in this setting.

ADR-related agents such as epirubicin, mitoxantrone, or pegylated liposomal doxorubicin show similar low efficacy [50–53]. Other systemic agents which were tested and showed very low activity, included 5-FU, capecitabine, gemcitabine, and irinotecan.

33.9.2 Combination Chemotherapy

Attempts to improve efficacy of chemotherapy by adding combinations of doublets, triplets, or more showed at times an increase in response but no real improvement in OS. Very few phase III trials are available and most data is from small phase II studies.

Doublets—Attempts to improve doxorubicin was attempted with several options. Cisplatin plus ADR showed RR ranging from 20 to 50 % [54]. Cisplatin plus capecitabine showed RR of 6 and 20 % in two studies [55, 56]. A more modern version of this combination uses oxaliplatin as the platinum backbone. A phase II trial combining oxaliplatin with capecitabine (XELOX) in 50 patients resulted in only 6 % RR [57]. A large Asian-based phase III trial randomized 371 patients to oxaliplatin and continuous infusion 5FU (modified FOLFOX4) versus single agent ADR. A small increase in OS was seen 4.97 months–6.4 months, which did not reach significance ($P = 0.07$) [58].

Gemcitabine-based doublets were explored with ADR, and reported RR of 20 % or less [59]. A phase trial II of 41 patients combined gemcitabine to liposomal doxorubicin. RR was 28 % with three patients reaching CR [60, 61]. No phase III data is available on the combination. The combination of GEMOX is considered standard of care in metastatic biliary cancer. A large retrospective analysis of 204 consecutive patients treated with GEMOX demonstrated 22 %RR and 66 % disease control rate [62]. Again no phase III data is available.

Triplets and more—The combination of three agents or more generally did not demonstrate a numerical increase in responses as compared to doublets. Numerous small single arms were published. A promising combination was of cisplatin/interferon alpha-2b/doxorubicin/fluorouracil (PIAF), especially in phase II. However, a phase III study compared PIAF to single agent ADR. RR increased from 10.5 to 20.9 %, respectively, but at the cost of increased toxicity with no statistically significant increase in OS [63]. A more recent retrospective publication of a modified PIAF regimen, reported a RR of 36 %; conversion to curative surgery in 33 % and a median OS of 21.3 months, but based on only 33 patients [64].

33.10 What Is Needed Next?

33.10.1 Improvements in Therapy of Unresectable HCC

The greatest need is the development of newer, more active drugs that have minimal hepatotoxicity. The antiangiogenics and the cell-cycle regulatory drugs appear to be attractive candidates.

33.10.2 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 1–2 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.

33.10.3 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 [65], some form of liver replacement therapy is still needed for the treatment of HCC that is based upon cirrhosis. Whether this is based upon 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.

33.10.4 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, Chap. 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 unrefrigerated 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.

33.10.5 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 [65].

The field of primary prevention (HBV vaccination, Chap. 19), early detection (surveillance screening of people at risk-cirrhosis), and the newer therapies (chapters on ^{90}Yttrium, growth modulators, antiangiogenics) 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.

33.10.6 Quantitation of Tumor Vascularity

The rapid incorporation into routine clinical practice of antiangiogenic 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.

33.10.7 Genomics and Proteomics of HCC

The rapidly expanding fields of both blood and tissue proteomics profiling and gene micro-arrays (Chaps. 5, 6, 7, and 8) 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 antiangiogenic 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.

33.11 Future Directions

33.11.1 Needs for TACE Standardization

There are many published reports of TACE and its methods. No trial has ever shown the superiority of 2, 3, or 4 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 [13]. 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 [8, 9], it would seem that either should represent the current TACE standard for future trials.

33.11.2 Combinations of TACE or Radioembolization 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. Results for just-published Bevacizumab plus erlotinib look even more exciting [22, 66].

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, or doxorubicin-TACE plus sorafenib, or intra-arterial ^{90}Yttrium plus sorafenib, or TACE plus bevacizumab and erlotinib. These combinations might result in the benefits of both tumor shrinkage as well as enhanced survival.

33.11.3 Adjuvant and Neo-adjuvant Therapies

The results of adjuvant chemotherapy trials for resection have been disappointing, apart from use of ^{131}I-lipiodol. A recent adjuvant trial with Sorafenib (STORM trial) had disappointing results. 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 towards offering this modality for multifocal tumors, there is a need for RCTs in the pre- or posttransplant setting. None have ever been published, even with chemotherapy. However, since only transplantation has the potential to simultaneously cure both the underlying liver disease as well as the tumor, there is a need for RCTs of chemotherapy, kinase inhibitors or antiangiogenics 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.

33.11.4 Newer Clinical Trials

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 (Sirspheres) plus sorafenib, gemcitabine, cisplatin plus sorafenib, some newer oral kinase inhibitors, new brachytherapies (^{32}P and ^{192}Ir), doxorubicin DEB, 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.

33.12 Medical Therapy Summary

Liver resection, transplantation or tumor ablation represent the only current therapies with potential for cure. Most HCC patients however, are not candidates for these three therapies at the time of diagnosis because of portal hypertension, poor liver function, tumor multifocality, portal vein tumor invasion/thrombosis (PVT) and/or comorbidities. TACE is the most commonly used nonsurgical treatment modality for these patients. It is the standard of care for patients with multiple lesions and well-preserved liver function, with or without branch PVT [67] and absence of metastasis. There is no tumor size limitation. Doxorubicin or cisplatin are well-studied, tolerated and partially effective chemotherapy agents in this setting. A bilirubin of <2 mg/ml and absence of ascites or minimal ascites seem to offer the safest

conditions. Drug-eluting beads can also be used, but have not yet been shown to result in superior survival to conventional TACE. The chemotherapy is often mixed in an emulsion with or without [68] Lipiodol (Ethiodol) and commonly used embolization materials include gelatin sponge particles (Gelfoam) or defined-size microspheres (Embosphere Microspheres), which are typically given as 100–300 μm spheres (and larger for shunting). Injecting the chemoembolization mixture as close to the artery feeding the tumor(s) as feasible, is thought to be associated with maximal antitumor effect and least hepatic parenchymal toxicity. Repeat treatments are typically given every 2–4 months, depending on blood counts, liver function tests and tumor size response and vascular responses on follow-up CAT scans. TACE in association with RFA has been reported to yield better outcomes than RFA alone. Tumor stabilization is likely a useful outcome and not a cause to switch therapy. By contrast, growth of tumor in a previously treated area of the liver is considered a treatment failure and cause for change of therapy. Typically, the choices at that point are radioembolization or Sorafenib.

^{90}Yttrium spheres regional therapy can be delivered by use of Theraspheres (Nordion) (a pure radiotherapy) or Sirspheres (Sirtex) (radioembolization or TARE, which offers lower radiation dose but greatly increased number of spheres per treatment). The two agents have not been directly compared, nor are survival results available for comparisons of ^{90}Yttrium spheres regional therapy with TACE. However, TACE needs to be used with caution in the presence of major branch PVT and not at all in presence of main stem PVT. By contrast, several papers have shown the relative safety of ^{90}Yttrium spheres in the presence of PVT. This is likely to make this therapy a first choice in presence of PVT. Thus, for patients with tumor progression following TACE, the choice is ^{90}Yttrium spheres or oral therapy with Sorafenib, since there is no trial data to indicate which is the superior choice [69]. For patients failing first line ^{90}Yttrium spheres therapy, the second line choice is Sorafenib.

Recently, external beam radiation therapy (EBRT) has been offered to patients who are surgically unresectable and cannot have other local therapy, such as major branch PVT, with encouraging response and safety data. However, this is in early phases of evaluation.

Patients failing TACE or ^{90}Yttrium spheres therapy or Sorafenib and who have good performance status, or who have metastasis, are often offered clinical trial enrollment with new agents, if their general performance status is satisfactory. Similarly, if they would otherwise have been offered TACE or ^{90}Yttrium spheres therapy or Sorafenib, but a clinical trial comparing any of those with a new agent is available, then enrollment in the trial may be reasonable, since none of those three modalities is curative in this setting. Further Reading, see Refs. [67–77].

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Targeted Therapies for Hepatocellular Carcinoma

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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/mitogenactivated 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 [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).

34.1 Molecular Pathways

34.1.1 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), hepatocyte growth

factor and its respective receptor (HGF/c-MET), and the cytokine transforming growth factor-β (TGF-β1) receptor 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].

34.1.2 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].

34.1.3 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].

34.1.4 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. When the PI3K/Akt/mTOR pathway is activated, PI3K cleaves phosphatidylinositol (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]. Approximately half of the cases with HCC had overactivation of the PI3K/Akt/mTOR signaling pathway [16].

34.1.5 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]. Approximately half of the cases with HCC had activation of the Wnt/β-catenin signaling pathway [17].

34.1.6 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, 18]. The PI3K/Akt pathway can also activate NF-κB; Akt phosphorylates numerous proteins and can also activate NF-κB [19]. Constitutively, active NF-κB has been found in some forms of cancer and has been associated with hepatocarcinogenesis [1, 20].

34.1.7 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, 21].

34.1.8 Angiogenic Targets: VEGFR, PDGFR, and FGFR

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. 34.1: Angiogenic Signaling Pathways) [3]. 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 [22].

Therapies that abrogate VEGFR signaling initially slow tumor growth and inhibit angiogenesis. Continuous treatment with anti-VEGFR agents, however, promotes the upregulation of activation of other proangiogenic signaling pathways, namely, PDGF/PDGFR and fibroblast growth factor ligands and receptors (FGF and FGFR) [23–26]. The FGF signaling pathway, which is comprised of 4 receptors (FGFR1-4) and over 20 ligands (FGF1-20), exerts activity via the intracellular Ras/Raf/MAP/ERK and PI3K/AKT/mTOR pathways. Dysregulation of the FGF/FGFR pathway has been implicated in promoting neoangiogenesis, therapy resistance, and disease recurrence [23–26].

34.1.9 Extracellular Matrix Changes

hanges 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, 21, 27].

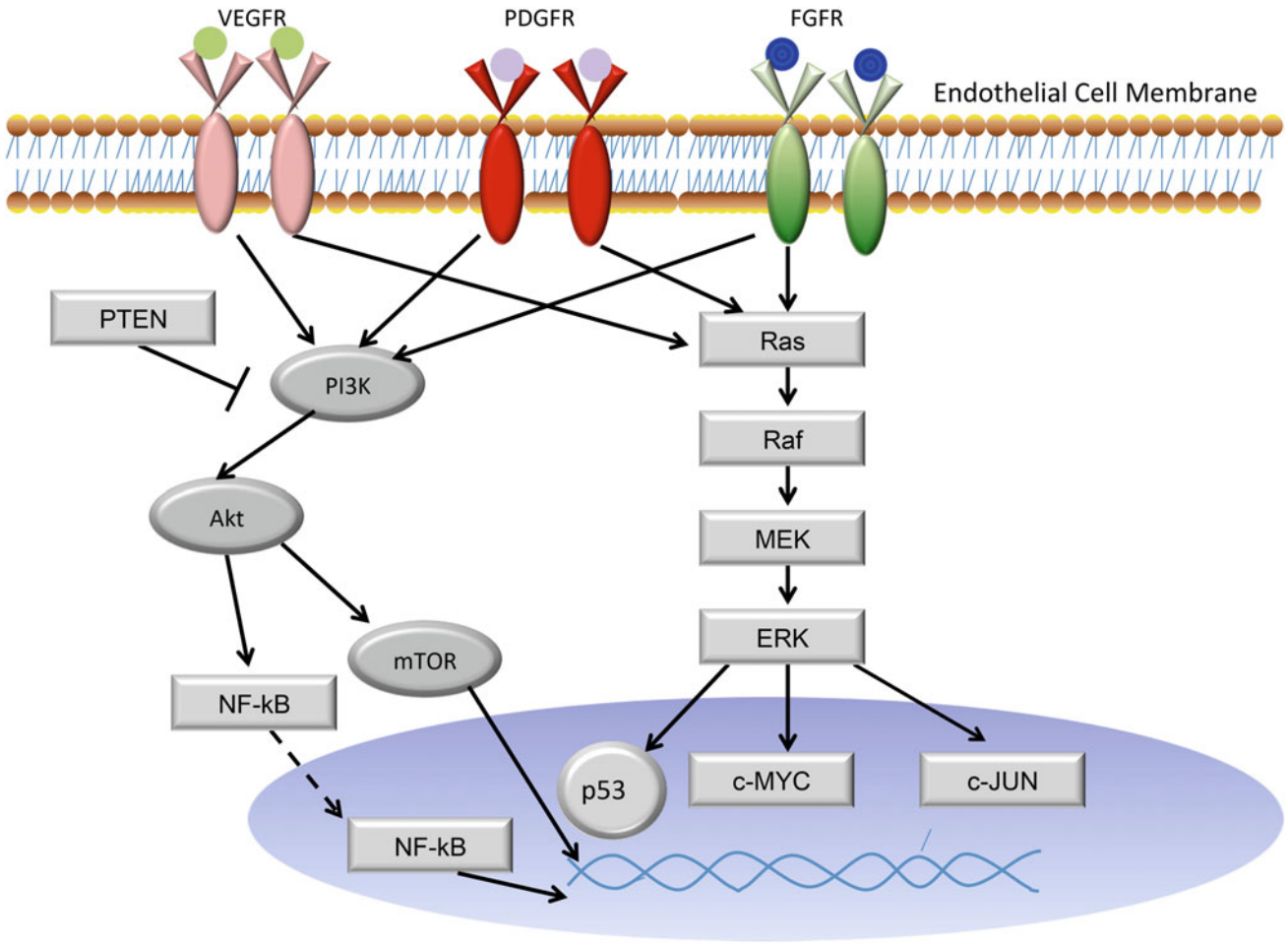

Fig. 34.1 Angiogenic signaling pathways for VEGFR, PDGFR, and FGFR

34.1.10 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 [21, 28, 29].

34.1.11 Immune Checkpoints

An optimally functioning immune system maintains a balance between tolerating normal cells with self-antigens, and eliminating pathogens and damaged cells [30, 31]. Cancer tumors modulate the signaling cascades of helper T cells to evade detection by the immune system [30, 31]. Understanding the signaling cascade can provide potential targets to reactivate the immune system and eradicate tumors.

Immune checkpoints that inhibit the immune system upon activation and that have been identified as targets for HCC includes the T lymphocyte associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD1) receptor with its respective ligands, programmed cell death 1 ligand 1 (PD-L1) and PD-L2. CTLA-4 and PD1 are expressed on helper T cells, and tumor cells express PD-L1 and PD-L2 [31–33].

34.1.12 Inflammation

Epidemiological studies suggested that use of anti-inflammatory agents, such as aspirin, lowers the risk of developing HCC versus nonuse [34, 35]. Elucidating the inflammatory pathways might lead to the development of novel therapies. Cyclooxygenase (COX) enzymes have been implicated in inflammation and hepatocarcinogenesis; aspirin inhibits COX-1 and COX-2, COX-2 is expressed at

low levels in normal tissue, and COX-2 is upregulated and overexpressed in HCC [36].

34.1.13 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, dysregulation of the PI3K/Akt/mTOR signaling cascade correlated with overactivity of other signaling pathways, such as EGFR, in over half the HCC cases [16]. 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]. For example, therapeutic inhibition of the VEGFR pathway in vitro leads to increased activation of the FGF/FGFR pathway, and ultimately, resistance to anti-VEGFR agents [23–26].

34.2 Rational Therapies

34.2.1 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.

34.2.1.1 Targeting EGFR

There are several pharmacologic agents in development that target one growth factor receptor in particular: 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 [19, 37]. 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 [19, 37, 38]. 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 [19, 37]. Gefitinib (Iressa) and erlotinib (Tarceva) are EGFR tyrosine kinase inhibitors, which compete with the ATP intracellular domain of EGFR inhibitors and prevent activation of the intracellular cascade [37]. Other EGFR tyrosine kinase inhibitors in clinical development include lapatinib (Tykerb) and AC480.

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 [39]. 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]. Erlotinib was recently evaluated in a phase III study, and will be further discussed in the polypharmacy section of this chapter.

34.2.1.2 Targeting HGF/c-MET

Agents that target the c-MET signaling pathway are also in development. One of the more exciting potential therapies for HCC within the last few years is tivantinib, a c-MET tyrosine kinase inhibitor that abrogates downstream Ras/Raf/MEK/ERK and P13/Akt/mTOR signaling pathways [40, 41]. Preliminary findings from a phase II clinical study suggest that biomarkers can potentially identify patients who are most likely to be responsive to tivantinib [42–44]. Patients (n = 107) who had experienced disease progression and/or intolerance to sorafenib or sunitinib were randomized into a tivantinib (360 mg twice a day) or placebo arm at a 2:1 ratio [43]. Notably, patients with MET-high tumors exhibited improved median overall survival with tivantinib versus placebo (7.2 vs. 3.8 months, respectively; hazard ratio = 0.38; P = 0.01). There was no statistically significant difference in overall survival between the tivantinib and placebo arms for patients with MET-low tumors (5.0 vs. 9.0 months, respectively; hazard ratio = 1.33, P = 0.50) [42, 43]. Although these preliminary findings suggest the use of c-MET as a predictive marker of

responsiveness to tivantinib, patients with MET-positive tumors need to be prospectively enrolled in phase III studies. There are 2 phase III clinical studies that are recruiting patients with diagnostically c-MET-high tumors; these studies will evaluate the efficacy and safety of tivantinib in the second-line setting [45].

Other therapies that target the HGF/c-MET pathway are in development and being evaluated in clinical trials, such as emibetuzumab, a monoclonal anti-MET antibody that targets the extracellular receptor. [24, 45–47]. Unlike tivantinib, however, biomarkers are not being integrated into these studies [45]. Other agents that target c-MET in addition to other signaling pathways will be further discussed in the multitargeted kinase inhibitors section.

34.2.1.3 Targeting Other Growth Factors

Other agents in development target IGF-1R, such as anti-IGF-1R antibodies (i.e., cixutumumab, BIIB002); these agents are currently being evaluated in phase I studies in combination with other therapies [45]. A therapy that targets TGF-β1R (i.e., galunisertib) is also in development [48].

34.2.2 Targeting Ras/Raf/MAP/ERK

Numerous therapies that abrogate the intracellular Ras/Raf/MAP/ERK signaling cascade are in development. For example, donafenib, a ras inhibitor, is currently being evaluated in phase I/II studies [45]. The downstream MAP protein is an important target to evaluate. For example, even in the absence of a Ras or Raf mutation, constitutively activated MEK has been reported in HCC cases [47, 49]. MEK inhibitors in development include selumetinib (AZD6244), refametinib (BAY 86-9766), and trametinib, and are under evaluation in phase II clinical trials [28, 45, 50].

34.2.3 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 [51]. Other therapeutic agents in early clinical development, such as alkylphospholipid perifosine, target Akt [52].

There are many agents in development that block the downstream effector, mTOR. The mTOR inhibitors in development include everolimus, temsirolimus, and sirolimus [1, 19, 53]. Everolimus and temsirolimus are currently approved for other tumor types. There are several phase I/II trials evaluating temsirolimus, either administered alone or in combination with other therapies [45].

The mTOR inhibitor that has reached the most advanced stage of development is everolimus, which was recently evaluated in a phase III study in a second-line setting [54]. Although sorafenib has provided benefit by extending the median overall median survival of patients with advanced HCC by approximately 2–3 months, sorafenib has been unable to extend survival to 1 year [55, 56]. There is an unmet need for additional therapies for advanced HCC in the second-line setting; after patients experience disease progression with sorafenib, there are no currently approved targeted therapies to slow or halt disease progression. Moreover, approximately 30 % of patients discontinued therapy because of sorafenib-associated adverse events [57]. Therefore, safe and effective therapeutic options to be administered in the second-line setting are an unmet need in the management of advanced HCC. The efficacy and safety of everolimus was assessed in a phase III study (EVOLVE-1) (n = 546) [54]. After treatment failure with sorafenib, patients were randomized into an everolimus (everolimus at 7.5 mg/day plus best supportive care) or placebo (placebo plus best supportive care) arm at a 2:1 ratio. The primary end point, improved overall survival, was not achieved; there was no statistically significant difference in median survival between the everolimus and placebo arms (7.6 vs. 7.3 months, respectively, hazard ratio = 1.05; P = 0.68). The most common severe (grade 3) and life-threatening (grade 4) adverse events in the everolimus arm were anemia (7.8 %), asthenia (7.8 %), and decreased appetite (6.1 %) [54]. Everolimus is still being evaluated in a phase II clinical study, although it will be evaluated in combination with sorafenib [45].

34.2.4 Targeting Wnt/β-Catenin

Pharmacologic agents in development that target the Wnt/β-catenin signaling pathway are in preclinical development. These include anti-Wnt antibodies, which disrupt activity of the downstream Wnt effector, β-catenin, and promote apoptosis in cancer cell lines [1, 58–62]. Other therapies in development include ICG-001 and PMED-1; these agents disrupt the interaction between β-catenin and the transcription regulator CREB-binding protein, and ultimately inhibit downstream signaling [52, 59, 63].

34.2.5 Proteasome Inhibitors

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) [64]. Apoptosis was induced only in HCC cells, whereas non-HCC hepatocytes did not exhibit apoptosis [64]. Proteasome inhibitors in development include bortezomib and oprozomib [65]. Proteasome inhibitors, in combination with other therapies, are under evaluation in phase II clinical studies [45].

34.2.6 Targeting Angiogenic Pathways: VEGFR, PDGFR, and FGFR

Because VEGFR and PDGFR stimulate proangiogenic pathways, pharmacologic agents that target these receptors can inhibit this process. A pharmaceutical agent in development is bevacizumab, an anti-VEGF antibody; by removing the VEGF ligand, the proangiogenic VEGFR signaling pathway should not be activated [21]. Although bevacizumab as a single agent exhibited activity in a phase II study evaluated patients with advanced HCC (i.e., a 13 % objective response rate was achieved), there are currently no plans for further development of bevacizumab as a single agent in phase III studies [47, 66]. The efficacy and safety of bevacizumab in combination with other therapies, however, is still under evaluation in ongoing phase II studies [45].

Ramucirumab (IMC-1121B), a fully human anti-VEGFR-2 monoclonal antibody, was recently evaluated in a phase III clinical study the second-line setting (REACH) [67, 68]. Patients (n = 565) were randomized at a 1:1 ratio into a ramucirumab (intravenous ramucirumab at 8 mg/kg plus best supportive care) or placebo (placebo plus best supportive care) arm in the second-line setting (i.e., experienced disease progression and/or intolerant to sorafenib) [68]. Patients in the ramucirumab arm failed to achieve the primary end point, improved overall survival; there was no statistically significant difference in survival between the ramucirumab and placebo arm (9.2 vs. 7.6 months, respectively; P = 0.14). The most common grade 3/4 adverse events in the ramucirumab arm included liver injury or failure (19 %), hypertension (12 %), and malignant neoplasm progression (6 %) [68]. Although ramucirumab alone failed to achieve improved survival as a single agent in a second-line setting, the efficacy and safety of ramucirumab in combination with other therapies is being investigated in ongoing clinical studies [45].

Other therapies that target proangiogenic signaling pathways include axitinib, a VEGFR-1,-2,-3 kinase inhibitor, and dovitinib, an FGFR3 kinase inhibitor [19, 45, 69, 70]. Therapies in development that target multiple proangiogenic signaling pathways will be discussed further in the multitargeted kinase inhibitors section.

Antiangiogenic therapies are shown in Fig. 34.2. Therapies that target other pathways are shown in Fig. 34.3.

34.2.7 Targeting Immune Checkpoints

Therapies that target the CTLA-4 and PD1/PD-L1 immune checkpoints are in development for HCC. Some of these agents have already exhibited efficacy against other malignancies; both ipilimumab (Yervoy), an anti-CTLA-4 antibody, and nivolumab (Opdivo), anti-PD-L1 antibody, have been approved by the FDA for melanoma [31, 71].

Antibodies that target CTLA-4 or PD1 abrogate activation of the inhibitory immune pathway. Anti-CTLA-4 antibodies (i.e., tremelimumab, ipilimumab) are currently under evaluation in clinical trials for HCC [31, 70]. The most advanced therapy for HCC that targets an immune checkpoint is nivolumab. In a recently reported phase I/II clinical study, nivolumab exhibited activity as assessed by reduction in tumor size in patients with HCC [72]. A phase III trial to evaluate the efficacy and safety of nivolumab in HCC has been registered [45].

34.2.8 Multitargeted Kinase Inhibitors

To date, the only multitargeted kinase agent to be FDA approved for the management of HCC is sorafenib. Over the last 5 years, other multitargeted kinase agents (i.e., sunitinib, linifanib, brivanib), have been evaluated; these agents failed to improve overall survival in phase III studies, and will be discussed in more detail below.

34.2.8.1 Sorafenib

Sorafenib (Nexavar) inhibits the Ras/Raf/MAP/ERK pathway, VEGFR-2 and -3, PDGFR-β, KIT, RET, and Flt-3 receptor tyrosine kinases [73–75]. 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 10.7 months (hazard ratio = 0.69; P < 0.001) [56]. Sorafenib was generally well tolerated. The most common (any grade) drug-related adverse events reported in 10 % or more of the sorafenib arm included diarrhea (39 %), fatigue (22 %), hand–foot skin reaction (21 %), rash/desquamation (16 %), alopecia (14 %), anorexia (14 %), and nausea (11 %) [56]. The most common grade 3/4 adverse events were hand–foot skin reaction (8 %) diarrhea (8 %), fatigue (3 %), hypertension (2 %), weight loss (2 %), and abdominal pain (2 %) [56]. 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

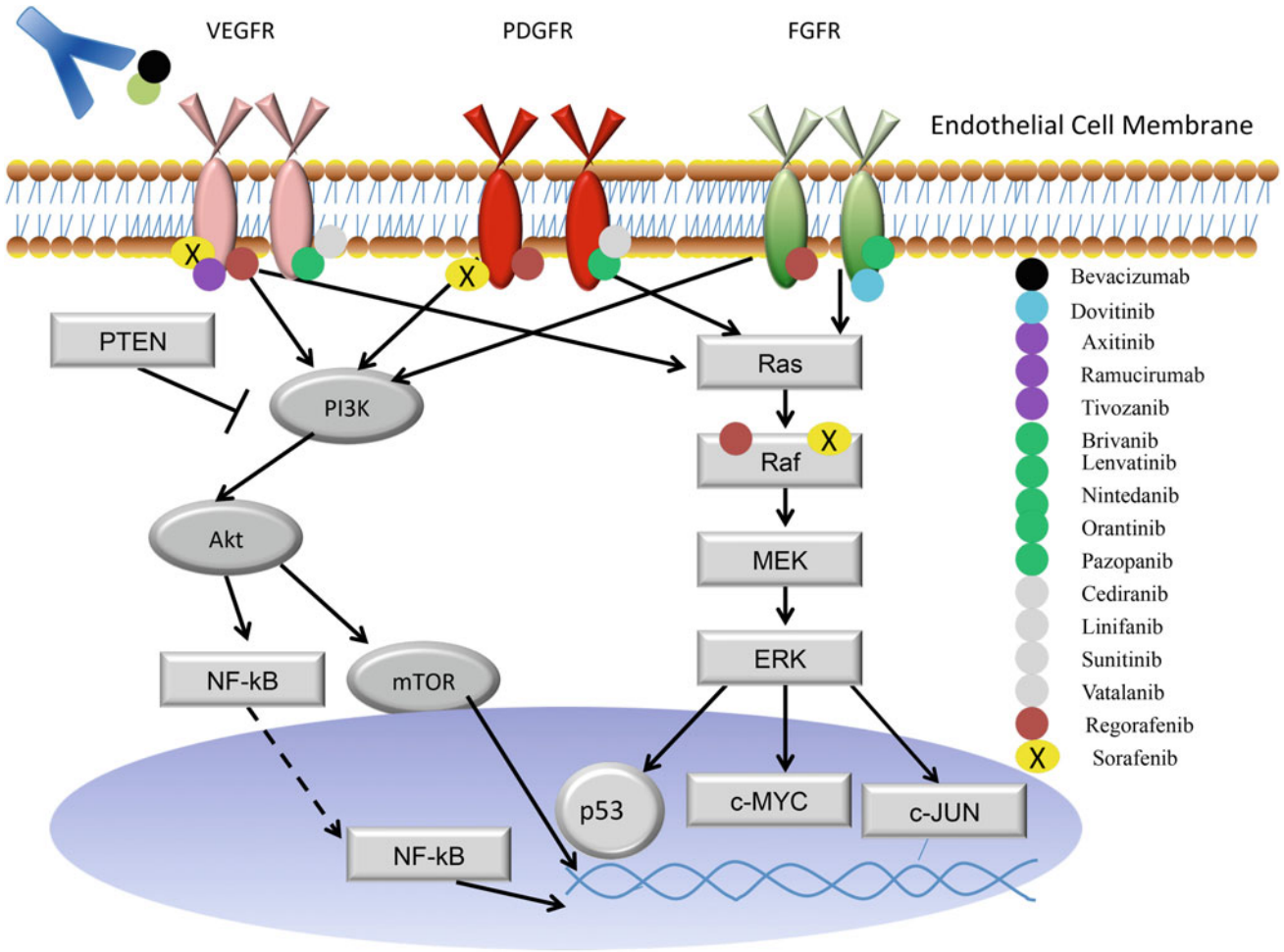

Fig. 34.2 Antiangiogenic therapies

targeted agent to reach the clinic for the treatment of HCC. Sorafenib has been integrated into the National Comprehensive Cancer Network (NCCN) guidelines; for patients who are not candidates for resection or a liver transplant, sorafenib is a treatment option [76]. Sorafenib is the only approved therapy for patients with metastatic HCC [76]. Moreover, within this treatment algorithm, sorafenib has a category 1 recommendation (i.e., high-level evidence and consensus among the NCCN panel members) for patients with Child-Pugh Class A [76].

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 [52]. Consensus guidelines by the 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 [76]. To further explore the role of sorafenib for patients with Child–Pugh class B, approximately 320 patients with Child–Pugh class B are being recruited to participate in a phase III study to evaluate the efficacy and safety of sorafenib [45].

The benefit of sorafenib has also been validated in another large (n = 226) randomized, placebo-controlled, phase III trial [55]. This trial was conducted in the Asia-Pacific region and many patients (73.0 %) had hepatitis B virus. Patients were randomized into a sorafenib or placebo arm at a 2:1 ratio [55]. Overall survival significantly improved among 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. Drug-related adverse events (any grade) reported by 10 % or more of the sorafenib arm included hand–foot skin reaction (45 %), diarrhea (26 %), alopecia (25 %), fatigue (20 %), rash (20 %),

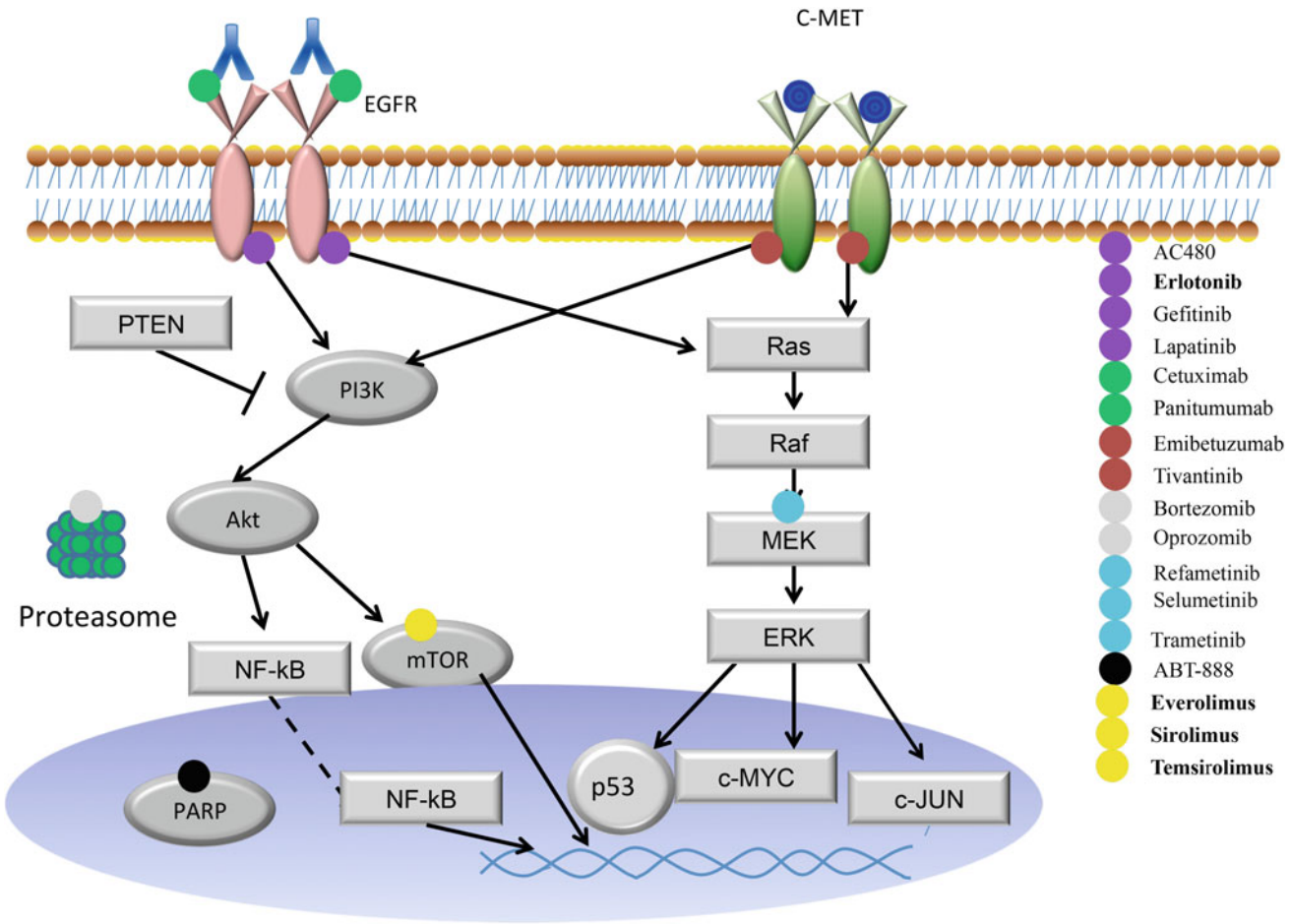

Fig. 34.3 Therapies with other molecular targets

hypertension (19 %), anorexia (13 %), and nausea (11 %) [55]. The most common grade 3/4 adverse events in the sorafenib arm included hand–foot skin reaction (10.7 %), diarrhea (6 %), fatigue (3.4 %), and hypertension (2 %) [55].

Managing sorafenib-associated adverse events remains challenging, and minimizing the toxicity of sorafenib might further improve the therapeutic index. Hypertension (any grade) was reported at an incidence of 19 % of the sorafenib arm in the pivotal phase III study [55], and grade 3/4 hypertension was reported at an incidence of 2 % of the sorafenib arms of both phase III pivotal sorafenib studies [55, 56]. Within the first few weeks of treatment with sorafenib, sorafenib-associated hypertension can occur [77]. Patients receiving sorafenib should be monitored weekly for hypertension [78]. Moreover, patients who develop hypertension should be managed with typical antihypertensive agents and if hypertension persists, sorafenib should be discontinued, either temporarily or permanently [78]. Hand–foot skin reaction is one of the most common (any grade) adverse events associated with sorafenib and is a dose-limiting toxicity [55, 56, 78, 79]. Hand–foot skin reaction typically occurs within the first few weeks of sorafenib therapy [78, 80]. Although suggestions have been made to reduce the likelihood of developing hand–foot skin reaction by minimizing exposure of a patient's hands and feet to hot water or excessive friction, and using topical agents if hand–foot skin reaction develops, there are no consensus guidelines or clinical trials to evaluate the management of hand–foot skin reaction [81, 82]. Severe hand–foot skin reaction may necessitate dose modification and/or discontinuation of therapy [78]. Approximately 30 % of patients have needed to discontinue therapy due to sorafenib-associated adverse events [57].

34.2.8.2 Sunitinib

Sunitinib (Sutent) inhibits VEGFR-1 and -2, PDGFR-α and -β, stem cell factor receptor c-KIT, and the FLT3 and RET kinases [2]. The efficacy and safety of sunitinib versus sorafenib was evaluated in an open-label phase III trial (n = 1074); overall survival was the primary end point [83]. Patients were randomized at a 1:1 ratio to receive sunitinib at

37.5 mg once a day or sorafenib at 400 mg twice a day. Patients in the sorafenib arm achieved superior overall survival; median overall survival in the sunitinib arm was 7.9 months overall, whereas the median overall survival for patients in the sorafenib arm was 10.2 months (hazard ratio = 1.30; one-sided $P = 0.9990$, two-sided $P = 0.0014$) [83]. The majority of adverse events reported in both study arms were mild (grade 1) to moderate (grade 2) in severity. A higher proportion of patients in the sunitinib arm (82.1 %) versus the sorafenib arm (74.2 %) had grade 3/ 4 adverse events. The most common grade 3/4 adverse events in the sunitinib arm included thrombocytopenia (29.7 %), neutropenia (25.7 %), and hand-food syndrome (13.3 %), whereas in the sorafenib arm this included hand-food syndrome (21.3 %) [83]. Due to lack of efficacy and safety concerns, the study was terminated early [83].

34.2.8.3 Linifanib

Another multitargeted kinase inhibitor in development is linifanib (ABT-869), a VEGFR and PDGFR tyrosine kinase inhibitor [13, 47].

In an open-label phase III study (LIGHT) ($n = 1035$), at a 1:1 ratio, patients were administered linifanib at 17.5 mg per day or sorafenib at 400 mg twice a day [84]. Linifanib failed to achieve the primary end point, overall survival; patients in the linifanib had a median overall survival of 9.1 months and patients in the sorafenib arm had a median overall survival of 9.8 months (hazard ratio = 1.046) [84]. Patients in the linifanib versus sorafenib arm had a higher frequency of grade ≥ 3 adverse events (85.3 % vs. 75.0 %, respectively; $P < 0.001$) and adverse events leading to drug discontinuation (36.3 % vs. 25.4 %, respectively; $P < 0.001$). The most common grade 3/4 adverse events experienced by patients in the linifanib arm included hypertension (20.8 %), palmar-plantar erythrodysesthesia syndrome (13.7 %), AST increased (12.2 %), and diarrhea (12.0 %), whereas the most common grade 3/4 adverse events experienced by patients in the sorafenib arm included palmar-plantar erythrodysesthesia syndrome (14.8 %), AST increased (12.5 %), and hypertension (10.6 %) [84].

34.2.8.4 Brivanib

Another multitargeted kinase inhibitor in development for HCC includes brivanib (AEE788)—an inhibitor of the FGFR-1, PDGFRβ, and VEGFR-2 pathways [21, 85, 86].

In a phase III noninferiority study (BRISK-FL study), at a 1:1 ratio, brivanib versus sorafenib was evaluated in the first-line setting [87]. Patients ($n = 1150$) were administered brivanib at 800 mg once a day or sorafenib at 400 mg twice a day [87]. Brivanib failed to achieve the primary endpoint, noninferior overall survival; the median overall survival of patients in the brivanib arm was 9.5 months versus 9.9 months in the sorafenib arm (hazard ratio = 1.06, with the prespecified margin upper limit for HR $\leq$ 1.08) [87]. The most common grade 3/4 adverse events experienced by patients in the brivanib arm included hyponatremia (23 %), AST increased (15 %), fatigue (14.5 %), hypertension (13.3 %), and hyperbilirubinemia (12 %), whereas the most common grade 3/4 adverse events in the sorafenib arm included AST increased (17 %) and hand–foot skin reaction (15 %) [87].

In a phase III study (BRISK-PS trial), the efficacy and safety of brivanib in a second-line setting was evaluated [88]. Patients who experienced disease progression with sorafenib or were intolerant to sorafenib ($n = 395$) were enrolled and randomized at a 2:1 ratio to a brivanib (brivanib at 800 mg per day plus best supportive care) or a placebo (placebo plus best supportive care) arm [88]. Patients receiving brivanib failed to achieve the primary end point, improved median overall survival; the median overall survival for patients in the brivanib arm was 9.4 and 8.2 months in the placebo arm (hazard ratio, 0.89; $P = 0.3307$) [88]. The most common grade 3/4 adverse events experienced by patients in the brivanib arm included hypertension (17 %), fatigue (13 %), and hyponatremia (11 %) [88].

34.2.8.5 Other Multitargeted Kinase Inhibitors

Other multitargeted kinase inhibitors in earlier stages of clinical development for HCC include the following: vatalanib, a VEGFR, PDGFR, and c-KIT tyrosine kinase inhibitor; cediranib, a VEGFR-1,-2, and -3 and PDGFR-α and -β kinase inhibitor; pazopanib, a VEGFR-1,-2,-3, PDGFR-α,-β, FGFR-1,-3, and c-kit inhibitor; and orantinib, a PDGFR, FGFR, and VEGFR inhibitor [13, 45, 47, 69, 89–91].

Other multitargeted kinase inhibitors, which are currently being evaluated in phase III studies, include the following: Regorafenib, a VEGFR-1, -2,-3, FGFR-1,-2, PDGFR, RET, kit, RAF-1, BRAF, and BRAFv600 inhibitor; cabozantinib, a VEGR2 and c-MET inhibitor; and lenvatinib, a VEGFR-1,-2,-3, FGFR-1,-2,-3,-4, PDGFR, RET, and kit inhibitor [45, 47, 92].

The mechanisms of action of the various molecularly targeted agents in development are summarized in Table 34.1.

34.2.9 Polypharmacy

Another strategy under evaluation to improve survival in advanced HCC is polypharmacy. Even if a molecular signaling pathway is successfully abrogated, because of cross-talk, other signaling pathways can be dysregulated; for example, inhibiting the VEGF/VEGFR signaling pathway activates the PDGF/PDGFR and FGF/FGFR proangiogenic pathways [23–26]. Therefore, to improve outcomes in HCC, combination therapies that can abrogate more than one

Table 34.1 Overview of mechanisms of action of pharmacologic agents

Agent	Mechanism of action									
	VEGF	VEGFR	PDGFR	FGFR	EGFR	mTOR	MEK	c-MET	Ras	c-kit
AC480 (BMS-599626)					•					
Axitinib[a,s] (AG-013736, Inlyta)		•								
Bevacizumab[b] (Avastin)	•									
Brivanib (BMS-582664)[s]		•	•	•						
Cabozantinib[c] (XL184, Cometriq)		•						•		
Cediranib (Recentin)[s]		•	•							
Cetuximab[d] (Erbitux)					•					
Donafenib									•	
Dovitinib				•						
Emibetuzumab (LY2875358)								•		
Erlotinib[e] (Tarceva)					•					
Everolimus[f] (Certican, Zortress, Afinitor, RAD001)						•				
Gefitinib[g] (Iressa)					•					
Lapatinib[h] (Tykerb)					•					
Lenvatinib[i] (E7080, Lenvima)[s]		•	•	•						•
Linifanib (ABT-869)[s]		•	•							
Nintedanib (BIBF 1120, OFEV)		•	•	•						
Orantanib (TSU-68)[s]		•	•	•						
Panitumumab[j] (Vectibex)					•					
Pazopanib[k] (Votrient)[s]		•	•	•						•
Ramucirumab[l] (IMC-112B, Cyramza)		•								
Refametinib (BAY 869766, BAY86-9766)							•			
Regorafenib[m] (Stivarga)[s]		•	•	•						•
Selumetinib (AZD6244)							•			
Sirolimus (Rapamune)						•				
Sorafenib[s,n](Nexavar)		•	•						•	•
Sunitinib[s,o] (Sutent)		•	•							
Temsirolimus[p] (Torisel)						•				
Tivantinib (ARQ197)								•		
Tivozanib		•								
Trametinib[q] (Mekinist)							•			
Vatalanib (PTK787)		•	•							•
Vandetanib[r,s] (Zactima, Caprelsa)		•			•					

[a]Approved for advanced renal cell carcinoma after failure of one prior systemic therapy
[b]Approved for the following: metastatic colorectal cancer; non-squamous non-small cell lung cancer; metastatic renal cell carcinoma; glioblastoma; and persistant, recurrent, or metastatic carcinoma of the cervix; platinum-resistant recurrent epithelial ovarian, fallopian, or primary peritoneal cancer
[c]Approved for progressive, metastatic medullary thyroid cancer
[d]Approved for squamous cell carcinoma of the head and neck and EGFR-expressing, K-Ras mutation-negative metastatic colorectal carcinoma
[e]Approved for non-small cell lung cancer and pancreatic cancer
[f]Approved for the following: advanced hormone receptor-positive, HER2-negative breast cancer; neuroendocrine tumors of pancreatic origin (PNET); advanced renal cell carcinoma after failure of treatment with sunitinib or sorafenib; renal angiomyolipoma and tuberous sclerosis complex
[g]Approved for first-line treatment of patients with metastatic non-small cell lung cancer whose tumors have epidermal growth factor receptor (EGFR) exon 19 deletions or exon 21 (L858R) substitution mutations as detected by an FDA-approved test
[h]Approved for HER2-positive breast cancer

[i]Approved for locally recurrent or metastatic radioactive iodine-refractory differentiated thyroid cancer
[j]Approved for EGFR-expressing metastatic colorectal carcinoma
[k]Approved for renal cell carcinoma and soft tissue sarcoma
[l]Approved for advanced gastric or gastro-esophageal junction adenocarcinoma, metastatic non-small cell lung cancer, and metastatic colorectal cancer
[m]Approved for metastatic colorectal cancer and gastrointestinal stromal tumor
[n]Approved for HCC and renal cell carcinoma
[o]Approved for renal cell carcinoma, pancreatic neuroendocrine tumors, and gastrointestinal stromal tumor after disease progression on or intolerance to imatinib
[p]Approved for advanced renal cell carcinoma
[q]Approved for melanoma with BRAF V600E or V600K mutations as detected by an FDA-approved test
[r]Approved for medullary thyroid cancer
[s]Multitargeted tyrosine kinase inhibitor

Table 34.2 Ongoing phase II and III clinical evaluating single therapies

Pharmacologic agent	Mechanism of action	Phase	*N*	End points
Axitinib	VEGFR-1,-2,-3 inhibitor	II	29	OS, PFS, QoL, safety, response
Brivanib	Multitargeted TKI; VEGFR-2; FGFR-1; PDGFRβ	III	414	OS, TTP, response, DCR, DOR, DCR, safety
Cabozantinib[a]	VEGFR-2, c-MET inhibitors	III	760	OS, PFS, response
Donafenib	Ras inhibitor	I/II	106	TTP, safety
Gefitinib	TKI; EGFR inhibitor	II	40	Recurrence-free survival, biomarkers, safety
Lenvatinib	Multitargeted TKI: VEGFR-1,-2,-3; FGFR-1,-2,-3,-4; PDGFR; RET, kit	III	954	OS, PFS, TTP, response, QoL
Nintedanib	FGFR, VEGFR, and PDGFR inhibitor	I/II	134	TTP, MTD, OS, PFS, response, safety
Nintedanib	FGFR, VEGFR, and PDGFR inhibitor	II	124	TTP, MTD, OS, PFS, response, safety
Nivolumab	Anti-PD-L1 antibody	III	726	TTP, OS, PFS, response
Regorafenib[a]	VEGFR-1,-2,-3; FGFR-1,-2; PDGFR, RET, kit, RAF	III	560	OS, TTP, PFS, DCR, response
Tivantinib	c-MET inhibitor	III	160	PFS, OS
Tivantinib	c-MET inhibitor	III	346	PFS, OS, safety
Temsirolimus	mTOR inhibitor	I/II	50	MTD, PFS, response, safety, DOR
Temsirolimus	mTOR inhibitor	II	25	Safety, response, circulating tumor cells
Tivozanib	VEGFR inhibitor	I/II	49	PFS, OS, response, safety

DCR disease control rate; *DOR* duration of response; *OS* overall survival; *MTD* maximum tolerated dose; *PFS* progression-free survival; *QoL* quality of life; *TTP* time to progression
[a]Agents will be evaluated in the second-line setting

signaling pathway is being explored in clinical studies. Another rational for combination therapy is that together, two or more therapies might work synergistically to modulate signaling pathways [83, 84]. For example, interferon-α (IFN-α) activates the JAK1/STAT1 pathway and induces apoptosis in HCC models. But when IFN-α is used in combination with aspirin, significantly more STAT1 is activated and more apoptosis is induced [93].

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. Post-transarterial chemoembolization (TACE) has been associated with an activation of proangiogenic signaling pathways, such as an upregulation and increase in VEGF and FGF levels [94, 95]. Strategies under evaluation to improve outcomes include administering sorafenib after TACE. In a phase III study, patients were randomized into a sorafenib or placebo arm at a 1:1 ratio after TACE. Sorafenib failed to improve survival after TACE; the investigators attributed this failure to an inadequate dose of sorafenib and/or a delay in the initiation of sorafenib therapy [96]. A high proportion of patients in the

sorafenib arm (73 %) required sorafenib dose reduction. Moreover, the median daily dosage of sorafenib in the TACE study was lower than the median dosage of sorafenib in the pivotal SHARP and the Asia-Pacific studies [55, 56, 96]. Patients in the TACE study received a median daily dosage of 386 mg sorafenib, whereas patients in the sorafenib arms of SHARP and the Asia-Pacific study received a median daily dosage of 797 and 795 mg sorafenib, respectively [55, 56, 96]. Further confounding the findings of this study, approximately 60 % of patients in the sorafenib arm of the TACE study did not initiate sorafenib until 9 weeks or more post TACE [96]. Administering sorafenib with TACE will continue to be evaluated, although different scheduling strategies will be used. For example, patients are being recruited for a phase III to evaluate the use of sorafenib after TACE, and sorafenib will be administered within 72 h of randomization. In another ongoing phase III study, TACE will be initiated within 2 weeks of receiving a stable dose of sorafenib [45].

Another strategy to improve outcomes was the administration of sorafenib post resection or ablation in patients with an intermediate-to-high recurrence risk, which was evaluated in a phase III study (STORM) [82]. Patients (n = 1114) were randomized into a sorafenib (400 mg twice/daily) or placebo arm at a 1:1 ratio [82]. The primary endpoint, recurrence-free survival, was not achieved; recurrence-free survival was similar between the sorafenib and placebo arm (33.4 months vs. 33.8 months, respectively; P = 0.26) [82]. Similarly, there was no statistically significant difference between treatment arms for time to recurrence and OS [82]. Discontinuation rates due to AEs were much higher in the sorafenib versus placebo arm (i.e., 24 % vs. 7 %, respectively) [82].

In a phase III trial (SEARCH study) (n = 720), patients were randomized into a sorafenib plus placebo arm or sorafenib plus erlotinib arm at a 1:1 ratio [97]. Median overall survival was similar across the sorafenib and sorafenib plus erlotinib arms (9.5 months vs. 8.5 months, hazard ratio = 0.929; P = 0.18). The most common grade 3/4 adverse events in the sorafenib versus sorafenib plus erlotinib arms, respectively, included fatigue (17.5 % vs. 17.7 %), hand–foot skin reaction (17.5 % vs. 10.2 %), diarrhea (11.8 % vs. 19.3 %), AST (11.8 % vs. 13.8 %), and hyperbiliruminemia (11.5 % vs. 11.9 %) [97].

A strategy under evaluation to reduce HCC involves the use of vaccines against hepatitis B virus in populations at high risk for acquiring this virus; preventing infection with hepatitis B virus (HBV) would reduce the likelihood of developing HCC [98]. Among patients who develop both HBV and HCC, a strategy to reduce the risk of recurrence has included the use of antiviral agents. Among patients seropositive for HBV, postoperative treatment with an antiviral regimen (adefovir dipivoxil plus lamivudine or entacavir) reduced HCC recurrence [99].

There are numerous ongoing studies evaluating the efficacy and safety of sorafenib in combination with other chemotherapeutic agents or other targeted therapies [33].

34.3 The Future

Although the last 5 years have been disappointing, with novel, targeted agents evaluated in phase III studies for HCC failing to meet their primary endpoint, OS, in both the first-line (i.e., sunitinib, linifanib, erlotinib plus sorafenib, brivanib) or second-line setting (brivanib, everolimus, ramucirumab) [54, 68, 83, 84, 87, 88, 97], there is hope to further improve outcomes in HCC. Notably, there are numerous ongoing clinical trials evaluating single (Table 34.2) and combination therapies (Table 34.3).

Table 34.3 Ongoing phase II and III combination trials with targeted therapies

Treatment	Phase	*N*	End points
Aspirin + lamivudine after surgery	III	112	Recurrence-free survival, OS, safety
Bevacizumab + erlotonib	II	44	PFS at 16 weeks
Bevacizumab + erlotonib (vs. sorafenib)	II	120	Response, safety, OS
Bevacizumab + floxuridine + dexamethasone	II	55	Response, safety
Brivanib + TACE	III	870	OS, TTDP, safety
Galunisertib (LY2157299) + nivolumab	I/II	100	MTD, PFS, DOR, OS, response
Ipilimumab + SBRT	I/II	100	MTD, response
Ramucirumab + Emibetuzumab (LY2875358)	I/II	70	Response, safety
Sorafenib + capecitabine + oxaliplatin	II	52	PFS, OS, tumor response, safety
Sorafenib + doxorubicin	II	170	TTP, OS, response, QoL, biomarkers
Sorafenib + doxorubicin	III	480	OS, TTP, PFS, response

(continued)

Table 34.3 (continued)

Treatment	Phase	*N*	End points
Sorafenib + everolimus	II	106	PFS, response, TTP, OS, safety
Sorafenib + gemcitabine + oxaliplatin	II	78	PFS, response
Sorafenib + mapatumumab	II	101	TTP, OS, PFS, DOR, safety
Sorafenib + melphalan	II	31	response, PFS, safety
Sorafenib + oprozomib	Ib/II	140	MTD, TTP, OS, PFS, response, safety
Sorafenib + oxilapltin + S-1	II	100	OS, time to recurrence
Sorafenib + pravastatin	III	323	OS, PFS, TTP, QoL
Sorafenib + refametinib	II	14	OS, DOR, Response, PFS, safety
Sorafenib + temsirolimus	II	106	PFS, OS, TTP, response, safety
Sorafenib + temsirolimus	II	28	OS, PFS, TTP, response, safety
Sorafenib + SBRT	III	368	OS, TTP, PFS, QoL, safety
Sorafenib + TACE	II/III	246	OS, TTP, tumor response, safety
Sorafenib + TACE	II	63	TTP, safety
Sorafenib + TACE	II	228	TTP, OS, response, safety
Sorafenib + TACE	III	240	OS, recurrence
Sorafenib + TACE	III	412	PFS, OS, TTP, safety, QoL
Sorafenib + TACE + radiation	I/II	30	TTP, PFS, PS, safety
Sorafenib + TRC105	I/II	39	MTD, PFS, response, safety
Sunitinib + TACE	II/III	190	OS, relapse-free survival, QoL, safety
Trametinib + capecitabine + fluorouracil + leucovorin	II	89	Response, safety, OS
Tremelimumab + MEDI4736	I/II	129	OS, DOR, response, safety
Veliparib (ABT-888) + temozolomide	II	49	PFS, OS, safety, biomarker, benefit rate

DCR disease control rate; *DOR* duration of response; *MTD* maximum tolerated dose, *OS* overall survival; *PFS* progression-free survival; *QoL* quality of life; *SBRT* stereotactic body radiation therapy; *TACE* transcatheter arterial chemoembolization; *TTDP* time to disease progression; *TTP* time to progression

To improve the outcomes of patients with advanced HCC, the underlying genetic and molecular signaling pathways needs to be further defined and elucidated. Although some aberrant signaling pathways promote the initiation of tumors, other signaling pathways associated with oncogene addiction sustain the tumor; identifying and abrogating a signaling pathway that sustains the tumor would be more likely to achieve optimal tumor reduction [48, 100].

It is essential to conduct a biomarker analysis in clinical trials to assess whether biomarkers might identify subsets of patients more likely to respond to therapy, and prospectively enroll these patients into a clinical study. Unfortunately, recently reported phase III studies evaluating new treatments for HCC did not incorporate or report findings from a biomarker analysis [43, 100]. Despite this shortcoming, the promising preliminary findings from the responsiveness of patients with MET-high tumors to tivantinib is promising [43]. Although this suggests that personalized medicine may finally enter HCC treatment algorithms, the initial findings need to be verified by prospectively enrolling patients with MET-high tumors into phase III studies.

With the integration of biomarkers and the continued evaluation of targeted therapies, 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.

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Radiation Therapy for Hepatocellular Carcinoma

35

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35.1 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 nondiscriminatory 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 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, have been powerful software programs, which enable conversion of CT or MRI datasets into three-dimensional "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 nonmalignant 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.

35.2 Physics of Radiation Therapy

35.2.1 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 = 2000 × 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 via apoptosis, via collision with a cell, transferring some of its energy to the cell. This interaction exchanges some energy to the cell, and the photon will be deflected itself 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 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 also can 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).

35.2.2 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.

35.2.3 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 datasets 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].

35.2.4 Fourth Dimension 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 fudicial markers placed near the tumor can control when the radiation beam is on or off. When treating in a part of the body (i.e., lung or liver tumors) that change 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.

35.2.5 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.

35.2.6 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.

35.2.7 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.

35.2.8 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 endocuritherapy, 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.

35.3 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, gama 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].

35.3.1 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 is 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.

35.4 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 coagulapathy 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].

35.5 Clinical Studies

35.5.1 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 3DCRT 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 3DCRT. 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 3DCRT 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 < or = 5 cm, or 2 nodules < or = 3 cm) 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 two 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 one 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 in ongoing [19].

Multicenter cooperative group trials have only been attempted by the Radiotherapy Oncology Group (RTOG) which predated 3DCRT and NTCP modeling, which now enable partial liver doses >90 Gy. The first, RTOG 83-19, tested the addition of ^{131}I antiferritin monoclonal antibodies to doxorubicin plus 5-fluorouracil to patients that had 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 transarterial chemembolization (TACE). 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].

35.5.1.1 External Beam Radiation (3D-CRT/IMRT) and TACE

External beam radiation therapy (EBRT) was used for unresectable HCC, in total doses greater than 35 Gy with TACE, for salvage of initial TACE failures [29–31]. Seong et al. [29] reported the use of 3D-CRT (mean tumor dose 44 Gy ± 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 Gy ± 7.9 Gy at 1.8 Gy/day [31]. Park et al. [30, 31] studied the same patient cohort as Seong, and determined a dose–response relationship existed, with dose groupings of <40 Gy, 40 Gy to 50 Gy, and >50 Gy. An autopsy study of seven patients after radiotherapy for HCC suggested viable tumor remained despite doses of 50–70 Gy [32, 33]. Using two-dimensional treatment planning to deliver external beam X-rays with TACE, Guo [33] 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.

Guo et al. [34] conducted 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 analyses of nine clinical variables and one treatment variable (irradiation) were performed employing 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 % vs. 28.1 %, $P < 0.05$). The overall survival rates in TACE plus irradiation group (64.0 %, 28.6 %, and 19.3 % at 1 year, 3 years, 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 et al. [35] retrospectively studied 203 patients who received TACE for unresectable HCC. None of the patients had tumor thrombus, lymph node involvement, or extrahepatic metastasis based on computed tomography (CT) scans of the chest and abdomen. Among these patients, 54 patients also received combination therapy with EBRT. Tumor RR, survival, and failure patterns were analyzed and compared between the two groups. Objective responses—complete response (CR) and partial response (PR)—on CT 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 year, 2 years, and 3 years, respectively, improved over the non-radiotherapy group rates of 59.6 %, 26.5 %, and 11.1 % at 1 year, 2 years, 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.

35.5.1.2 External Beam Monotherapy

Challenges in the use of EBRT for HCC are many; however, successes are being realized with the use of image-guided radiotherapy (IGRT) to assist in the delivery of 3D-CRT, IMRT, and stereotactic body radiotherapy (SBRT), along with respiratory motion compensation and tumor visualization [36, 37].

Kim et al. [38] used 3D-CRT to treat unresectable HCC patients where TACE was ineffective or unsuitable, and to determine whether tumor response and 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 through the use of 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 %) patients, no response in 28 (37.1 %) patients, and progressive disease in 4 (8.6 %) patients. Of 41 patients with PVT, the PVT responses were CR in 4 (9.7 %) patients, PR in 12 (29.3 %) patients, NR in 20 (48.8 %) patients, and PD in 5 (12.2 %) patients. The median survival times were 18.0 and 20.1 months in the primary tumor and the PVT responders (CR + PR), respectively, were longer than the 6.8 and 7.2 months in the primary tumor and the PVT NRs (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 where TACE is ineffective or unsuitable.

Liu et al. [39] 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. The mean age was 62 years, ranging from 34 to 88 years. Eastern Cooperative Oncology Group (ECOG) performance status was 0 in 10 patients, 1 patient in 19 patients, and 2 patients in 15 patients. Child-Pugh classification was A in 32 patients and B in 12 patients, with 14 patients having main PVT. 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 patients of 44 patients, yielding a response rate of 61.4 %. The survival rates at 1 year, 2 years, 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.

The use of proton beam radiotherapy represents a different type of energy than photons that, by physical characteristics, can achieve superior dose deposition compared to 3D-CRT [40, 41].

35.5.1.3 External Beam Radiotherapy for Portal Vein Thrombosis

Several investigators have used 3D-CRT and SBRT successfully to treat PVT tumors and not the primary HCC lesions. Overall the response rate is approximately 80 % with very few side effects.

Potentially transplantable patients can benefit from RT as a bridge to transplant while on the wait list. In stages B and C, RT has efficacy in situations where TACE has been ineffective or is unsuitable. This is particularly important in patients with PVT where TACE is contraindicated, and where transarterial radioembolization (TARE) may not be possible or is ineffective [36, 37].

35.5.1.4 Proton (External Beam) Radiotherapy

Proton beam radiation therapy (PBT), referred to as "protons," has been used with success for treatment of HCC as reported in most published data from Japan. A fundamental difference between X-rays of traditional EBRT, and protons, is that protons carry a charge, have mass, and can be delivered into deep tissues with lower radiation deposition above and below the target. X-rays, where photons are electromagnetic waves and have no charge or mass, release nearly all of their energy within the tumor. Because of increased control of radiation dose deposition at any depth in the body, there has been intense interest in using PBT for treatment of HCC.

Currently, proton accelerators are of limited availability (about 20 total) in the United States and the same number outside the US because of the enormous cost of constructing the accelerators ($100 million USD per facility). A proton accelerator requires a cyclotron onsite. Clinical use of protons is mostly for pediatric tumors, and adult CNS, spinal cord, ocular, skull base, 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 [42].

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 et al. [43] reported a 3-year actuarial local control rate of 93 %. Matsuzaki et al. [44] reported the use of protons for 24 patients failing TACE for HCC, and found tumor response in >90 % of these lesions.

It is not known whether SBRT or PBT is superior or equivalent in outcomes of HCC patients [45]. Currently, only one 2a evidence exists that supports any form of radiation in HCC; however, combined with the retrospective reports of hundreds of patients, there is a significant amount of evidence supporting the use of RT in all stages of HCC [40, 41]. PBT may become more common as new facilities currently planned worldwide become operational.

35.5.1.5 Stereotactic Body Radiotherapy (SBRT) Studies

A strong interest in pursuing SBRT for treatment of HCC is apparent due to the increased ability of SBRT to spare normal liver tissue from receiving tolerance doses of radiation. Four prospective studies and four retrospective reports are available from 2006 to 2011 that involve a range of 80 patients to 60 patients. The positive outcomes in all stages of HCC are proven with a wide array of fraction sizes and total doses. Three of the studies used at least five different fractionation schedules adjusted for Child-Pugh A or B classes. One-year survival ranged from 48 to 79 % in these heterogeneous groups [46–48].

SBRT was studied in a phase I/II trial of mixed neoplasia in the liver, which included one HCC patient. Herfarth et al. [49] 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.

Wu et al. [50] used SBRT combined with TACE in 94 patients with cirrhosis and HCC. A total 63 patients had Okuda stage I lesion and 31 patients had stage II lesion. 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 weeks to 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, 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 et al. [51] completed a phase I study of individualized SBRT for unresectable HCC and intrahepatic cholangiocarcinoma (IHC) not suitable for standard therapies. Six fractions of SBRT were delivered over 2 weeks, 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 (9–1913 mL). The median dose was 36.0 Gy (24.0–54.0 Gy). No radiation-induced liver disease or treatment-related grade 4 or grade 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.

35.5.2 Brachytherapy

35.5.2.1 ^{131}I-lipiodol

Most commonly, brachytherapy for HCC has been accomplished by hepatic artery infusion of ^{90}Y-embedded microspheres, or ^{131}I-lipiodol (^{131}I). 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 nonradiation embolic therapy in the liver, containing 38 % iodine by weight was a logical choice to add a radioisotope. In animal studies, ^{131}I 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 by Ho, there were 14 studies between 1985 and 1997, with more than 400 patients having received this therapy [52, 53]. Most patients with unresectable HCC were treated for amelioration of symptoms; response rates were 25–70 % in uncontrolled studies. Raoul et al. [53, 54] reported a multicenter randomized study of patients with PVT from HCC who received 10–100 Gy in 1–5 injections and had better survival than the control (untreated) group. In a separate prospective trial of 142 patients with unresectable HCC, randomization was to ^{131}I 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.

^{131}I was tested in the postoperative adjuvant setting in a prospective randomized trial by Lau et al. [55], which was stopped early. Randomized patients after resection in the experimental arm received ^{131}I (1850 MBq in a single dose) or no further therapy (control group). Interim analysis of 21 treated patients and 22 control patients showed a statistically significant decrease in recurrence (28.5 % vs. 59 %), and improved median disease-free survival (57.2 months vs. 13.6 months) for the treated patients.

Lau et al. [55] 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 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 1850 MBq dose of ^{131}I or no further treatment (controls). Comparison of rates of recurrence, and long-term disease-free and overall survival (primary endpoints) between the two groups, by intention-to-treat, was completed on 43 patients totally (21 radiation group, 22 controls). ^{131}I had no significant toxic effects. During a median follow-up at 66 months, (range, 3–198 months) there were 10 (47.6 %) recurrences among the 21 patients in the adjuvant treatment group, compared with 14 (63.6 %) recurrences 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 the use of adjuvant intra-arterial ^{131}I after curative liver resection provides a survival benefit of disease-free survival and overall survival, although the difference became statistically insignificant 8 years after randomization.

35.5.2.2 ^{90}Y Microspheres (Yttrium-90)

Radioembolization (RE) is a form of brachytherapy during which microspheres containing Yttrium-90 (^{90}Y) are implanted into hepatic tumors via the hepatic artery. The radiation is permanently bound to the microspheres, which do not migrate out of the liver tumors. Almost pure beta radiation is delivered within an effective range of only 2.5 mm from the microsphere, thus sparing normal adjacent liver tissue from damage. The half-life is 64 h with all of the effective radiation delivered by 14 days post implant [56–58].

The rationale for microsphere treatment with infusion of a sphere charged with ^{90}Y is that ^{90}Y will undergo beta decay with energetic electrons thereby penetrating only 2–8 mm, over a half-life of 64 h. Microspheres, which range in diameter from 20 to 40 microns, will become embedded within the tumor vasculature, but because the end arterioles are <10 microns in diameter, the microspheres will not pass into the venous circulation. The lungs are the next arteriole bed, which would capture the spheres (Figs. 35.1 and 35.2). Pulmonary tolerance to radiation is roughly half (<20 Gy) that of the liver and unintentional deposition of microspheres with ^{90}Y led to deaths in past trials [59, 60]. Arteriovenous shunts in the liver that would allow free passage of microspheres into the venous system and then to the lungs were not readily apparent on angiogram. Therefore, patient screening involves detailed hepatic angiographic mapping coupled with nuclear imaging using albumin tagged with a gamma emitter technecium-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 to 15 % of the dose appears in the lungs, a dose reduction of microspheres is attempted, or the procedure is aborted [61–79]. Infusion of the entire liver can be accomplished in a single infusion; however, this procedure will increase toxicity versus a sequential lobar approach, with a 4-week interval between infusions [61].

There is recent evidence that it is safe to add ^{90}Y as treatment for PVT cases in situations where TACE is contraindicated. As part of a single center, prospective longitudinal cohort study, Salem et al. [62] treated 291 HCC patients with ^{90}Y to assess clinical outcomes. RR and TTP were determined using World Health Organization (WHO) and European Association for the Study of the Liver (EASL) guidelines. Five hundred twenty-six treatments with ^{90}Ywere administered (mean: 1.8, range: 1–5). Toxicities included fatigue (57 %), pain (23 %), and nausea/vomiting (20 %); 19 % exhibited grade 3/4 bilirubin toxicity. The 30-day mortality rate was 3 %. Survival times differed

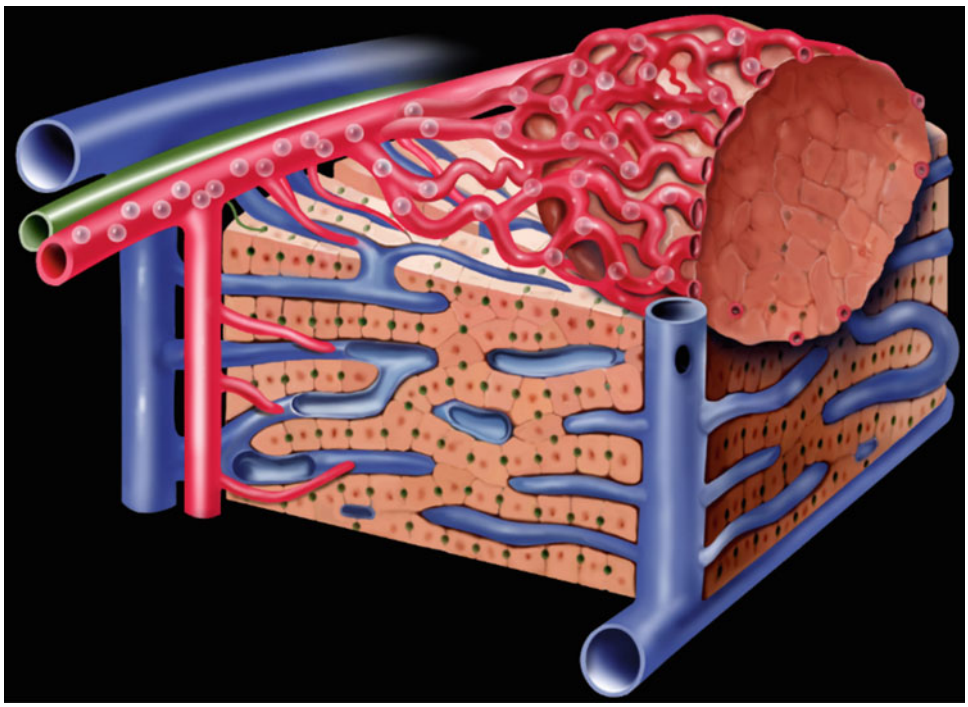

Fig. 35.1 Illustration of the arterial plexus of abnormal vessels recruited by hepatocellular cancers and the route 90Y-microspheres take to embed into the tumor. The beta radiation emitted only penetrates 3–4 mm from each microsphere sparing the adjacent normal liver tissue beyond the tumor

Fig. 35.2 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

between Child-Pugh A and B patients (A:17.2 months, B:7.7 months, $P = 0.002$). Child-Pugh B patients with PVT survived 5.6 months (95 % CL:4.5–6.7). The results showed that Child-Pugh A patients, with or without PVT, benefited most from ^{90}Y treatment. Sangro et al. [63] conducted a multicenter analysis to evaluate the main prognostic factors driving survival after RE using ^{90}Y microspheres in patients with HCC. Three hundred twenty-five patients were administered 1.6 GBq infusion between September 2003 and December 2009. Patients were Child-Pugh class A (82.5 %), who had underlying cirrhosis (78.5 %), and had good ECOG performance status; however, many had multinodular disease (75.9 %) invading both lobes (53.1 %) and/or PV occlusion (13.5 % branch; 9/8 % main). Over half of the patients had advanced Barcelona Clinic Liver Cancer (BCLC) staging (BCLC C, 56.3 %) and one-quarter had intermediate staffing (BCLC B, 26.8 %). The median overall survival was 12.8 months (95 % confidence interval, 10.9–15.7), which varied by disease stage (BCLC A, 24.4 months [95 % CI, 18.6–38.1 months]; BCLC B, 16.9 months [95 % CI, 12.8–22.8 months]; BCLC C, 10.0 months [95 % CI, 18.6–38.1 months]). Survival varied by ECOG status, hepatic function (Child-Pugh class, ascites, and baseline total bilirubin), tumor burden, and presence of extrahepatic disease. Overall survival diminished in patients with PV occlusion (branch or main) compared with those with patent vessels (10.0 months: 95 % CI, 6.5–11.8 vs. 15.3 months; 95 % CI, 12.4–18.4; $P = 0.003$), with no significant difference in survival between patent portal vein and branch occlusion ($P = 0.124$). Data from both studies describe ^{90}Y as a potential treatment option to patients with HCC. Although sorafenib is currently the standard of care for advanced HCC, these studies demonstrate that the antitumoral effect of ^{90}Y should be further studied. Advanced HCC patients with PVT may represent a select cohort where combinatorial therapy of ^{90}Y with sorafenib therapy may significantly improve outcome.

The most common nonsurgical approaches for the treatment of localized hepatocellular carcinoma remain TACE and TARE [45]. TARE has no macroembolic effect [65], can be safely applied to patients with PVT, and offers a median survival in thee range of 6–11 months [65–68]. Similar results (6.5–10.7 months) were also produced in phase III clinical trials of sorafenib with the same group of patients [70, 71]. Interestingly, HCC patients with PVT (branch or segmented), survival increased, 10–14 months [64–66]. With a potential to induce intense tumor responses, TARE has moved to the forefront of therapy to reduce tumor burden within acceptable limits for liver transplantation, to render nonoperable patients operable, or to simplify surgery. The United Network for Organ Sharing (UNOS) downsizing from T3 to T2 was realized more with TARE than with TACE (58 % vs. 31 %, $P = 0.023$). [74] Radiation lobectomy—contralateral lobe hypertrophy as a result of injection of a high activity of ^{90}Y in a lobar hepatic artery—and atrophy of the irradiated lobe after TARE may be a valuable contribution to resectability [75]. Inarrairaegui et al. [76] reported that in a group of 21 UNOS T3 stage patients, 29 % were moved to forefront surgical treatment or transplantation with a 3-year survival rate of 75 %, comparable with the survival in patients with early stage disease who are treated radically at the time of diagnosis. Chow et al. [77] conducted a multicenter, open-label, single arm, Phase II study (NCT0071279) to evaluate the safety and efficacy of sequential TARE-sorafenib in patients with HCC

not amenable to curative therapies. Sorafenib 400 mg, twice daily, was initiated 14 days post TARE with ^{90}Y microspheres given as a single procedure. Twenty-nine patients with BCLC stage B (38 %) or C (62 %) HCC received a median of 3.0 GBq ^{90}Y followed by sorafenib (median dose/day, 600.0 mg; median duration, 4.1 months). Twenty-eight patients experienced $\geq$ toxicity; 15 (52 %) grade $\geq$ 3. Disease control was 100 and 65 % in BCLC stage B and stage C, respectively. Two patients (7 %) had sufficient response to enable radical therapy. Median survivals for BCLC stage B and stage C were 20.3 months and 8.6 months, respectively. In the multicenter SORAMIC trial, Ricke et al. [78] randomized 40 patients to TARE with ^{90}Y microspheres followed by sorefenib (n = 20) or sorefenib only (n = 20). Eligible patients were stratified by presence or absence of a PVT and randomly assigned in a 11:10 ratio to receive either sorafenib in combination with ^{90}Y microspheres or sorafenib alone. Patients were followed at 2-month intervals for a minimum of 24 months or until death. Sorafenib was given continuously until tumor progression or the emergence of drug-related adverse events (AEs), which required discontinuation after two dose reductions. All patients randomized to the ^{90}Y microspheres arm had a pretreatment assessment 1 to 2 weeks earlier to plan the selective delivery of the ^{90}Y microspheres in each liver lobe. This study represented the first formal prospective assessment of the toxicity of a combined treatment regimen of ^{90}Y microspheres and sorafenib. Data from the study indicated that sorafenib initiated 3 days after the last radioembolization procedure was generally well tolerated compared with sorafenib alone.

This ever-expanding body of level 2 evidence has vaulted TARE into the guidelines of the European Society for Medical Oncology (ESMO), the European Society of Digestive Oncology (ESDO), and the National Comprehensive Cancer Network (NCCN); however, not yet in the guidelines of the European Association for the Study of the Liver (EASL), the European Organization for Research and Treatment of Cancer (EORTC), or the American Association for the Study of the Liver Diseases (AASLD).

A consensus panel [80] provided category 2a consensus evidence and guidelines for employing internal liver radiotherapy with radioactive microspheres. One of its purposes was to standardize the indications, techniques, multimodality treatment approaches, and dosimetry to be used for ^{90}Y microsphere hepatic brachytherapy. Members of the Radioembolization Brachytherapy Oncology Consortium (REBOC) comprised an 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 by REBOC 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. REBOC 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 [81–83] were the first investigators to perform microsphere clinical trials in humans. During the early 1960s, most patients had metastatic carcinoid or colorectal cancers. The pioneering work of Ariel and Simon was with composite spheres and ^{90}Y but their treatment procedures for screening, infusion, and posttreatment imaging are largely intact in modern clinical practice [61, 84–93]. Two microsphere devices are available in the US: the glass microsphere (TheraSphere®) and resin-based sphere (SIR-Spheres®). Both are similar in size, and isotope (^{90}Y), but have some important differences in delivery and physical characteristics [94] (Table 35.1). Both began in clinical trials in the late 1980s and have been used in thousands of patients since, mostly with colorectal metastases, but sufficient HCC

Table 35.1 Comparison of radioactive microsphere agents

Parameter	Glass	Resin
Size (median)	25 microns	32 microns
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	2500 Bq	50 Bq
Indication(s)	HCC (USA) HCC and colon (Canada)	Colon (USA) All tumor types (Europe, Asia)
Regulatory status (United States FDA)	Humanitarian device exemption (HDE) HCC only	Premarket 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 have been treated to make some observations [59, 69, 85, 88, 89, 95–113].

Carr et al. [98, 105] presented a report of a phase II trial of glass microspheres via lobar approach, with a nominal target dose of 135 Gy and a quality of life companion study [99, 114]. Carr also statistically compared survival of published untreated Okuda I and II patients [115–117] to his study cohort [99, 105]. 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 (360–1012 days) 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 one 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, [118] and 16 patients by Soulen [119] were very similar to those reported by Carr, with elevated enzymes, nausea, and fatigue the most frequent common toxicity grade 2 or grade 3 findings. The dose delivered was different in all three studies; Kennedy [118] delivering median dose of 149 Gy (128–174 Gy) to the whole liver with a 9-month survival of 75 %, Soulen [117] a mean of 128 Gy (97–182 Gy), and Carr at 133 Gy [99].

35.5.2.3 Additional Phase I-II ^{90}Y-Microsphere Trials in HCC

Lau et al. [96] 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 et al. [95] 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 mCi to 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 1580 Gy. The residual tumors were resected in four patients and in two of these patients no residual tumor was found and in the remaining two 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 et al. [59] 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 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 eleven 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 week; the median survival was 54 week. 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 et al. [120] 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 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 EASL, the response rate was 70 %. The AEs were highest in patients with main PVT and cirrhosis. There were no cases of radiation pneumonitis. Kaplan–Meier survival varied depending on the 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 noncirrhotic, 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, [121–125] 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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Psychosocial Issues in Hepatocellular Carcinoma

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36.1 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 et al. [3] found in a sample of over 4000 cancer patients that 25–43 % reported significant distress. 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 health care providers. Fallowfield et al. [4] found that only 29 % of oncology patients who exceeded the cut-off score on a distress instrument were identified by their physicians as being distressed. 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]. Cancer centers 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 methods can be employed to complement conventional medical 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. 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].

36.2 The Role of Behavior in the Development of HCC

HCC is the sixth most common cancer 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.

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 are secondary to HBV and HCV infection followed by NASH and alcohol abuse, 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) may also contribute to more rapid disease progression and medical complications once diagnosed with HCC [28–30]. 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) 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, as well as infection with HCV, been found to have a synergistic effect in the development of HCC [41, 44, 46, 47, 53]. In two studies, the combination of alcohol and tobacco increased risk for HCC 5.6–7.2 times when compared to cirrhotic patients without these risk factors [41, 46]. Studies from Taiwan and Japan have also replicated these results [53, 54].

The most common environmental risk factor is the exposure to aflatoxins. Aflatoxins, formed by certain Aspergillus species, are frequent 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 including smoking cessation as well as further research and education regarding the role of environmental and/or occupational factors that may lead to HCC are critical in the prevention of this cancer.

36.3 The Role of Inflammation and HCC

Inflammation is a hallmark of immunological responses to invading microbes, but has been implicated in a number of major diseases, including inflammatory bowel disease, obesity, diabetes mellitus and several cancers. Chronic inflammation has been linked with specific types of cancer, particularly those associated with viral infection or an

inflammatory response, including hepatitis- and NASH-associated HCC. Although some chronic diseases have long been acknowledged to increase the risk of malignancies, only more recently has chronic inflammation been hypothesized to be a key factor in their development. There is increasing evidence that psychosocial factors directly contribute to the development and maintenance of chronic inflammation. While depression may contribute to increase levels of circulating pro-inflammatory cytokines, inflammation conversely may itself act on the brain to induce depressive symptomatology. Thus, interventions targeting the depressive symptoms associated with HCC may also disrupt the chronic inflammatory cycle and its resultant disease processes.

Virchow hypothesized in 1863 that tumors originated at sites of chronic inflammation within the human body and identified the role of inflammation in carcinogenesis when he noticed the presence of leucocytes in neoplastic tissue and suggested that the "lymphoreticular infiltrate" reflected the origin of malignancies where inflammatory processes occurred [61, 62]. His claim was not investigated for more than a century. Recently, researchers have begun examining the hypothesized relationship and studying the connection between chronic inflammation and cancer. Epidemiological studies have demonstrated that chronic inflammation predisposes individuals to a variety of cancers [61, 62]. About 25 % of all deaths from cancer worldwide are attributable to underlying infections and inflammatory responses [63]. Chronic infection and inflammatory responses are known to have associations with the development of certain cancers, such as the human papilloma virus (HPV) and its relationship to cervical cancer, or the infection of hepatitis B and C viruses leading to HCC [64]. Increased risk of tumor growth is associated with chronic inflammation caused by various microbial infections and autoimmune diseases (e.g., inflammatory bowel disease and the risk of colon and colorectal cancers) [65]. Chronic inflammation contributes to a tumor-promoting environment through various avenues that may include cellular transformation, proliferation and survival of malignant cells, development of angiogenesis and metastasis, reduction of adaptive immune responses, as well as tumor responses to chemotherapeutic drugs and hormones. The inflammatory response and resultant tumors may be conceptualized as wounds that do not heal [66].

The role of chronic inflammation in the development of cancer involves the contributions of various inflammatory cells, mediators, and signaling pathways in cancer genesis and inflammatory mediators which include chemokines and cytokines in tumor tissues, tissue remodeling, and angiogenesis. The prime endogenous promoters include transcription factors such as nuclear factor-kappa B (NF-kB) and signal transducer activator of transcription-3 (Stat3), as well as major inflammatory cytokines, such as interleukin beta (IL-1β), interleukin 6 (IL-6), interleukin 23 (IL-23), and tumor necrosis factor alpha (TNFα) [67–70]. TNFα was the first factor isolated as an anticancer cytokine. At dysregulated levels within the immune system, its presence mediates a variety of diseases and has also been demonstrated to be a major predictor of inflammation [71, 72]. Several pro-inflammatory cytokines have been related to tumor growth, indicating that inflammation is associated with carcinogenesis [61, 73]. These include IL-1, IL-6, IL-8, and IL-18 and are involved in different steps of tumor initiation and growth. Specifically, Negaard et al.demonstrated that individuals with hematological malignancies have increased bone marrow micro-vessel density as well as elevated levels of IL-6 and IL-8, possibly contributing to the malignant phenotype [74]. Chemokine proteins play several roles in cancer progression, including angiogenesis, inflammation, and cell recruitment and migration and play a central role in leucocyte recruitment to sites of inflammation. Most tumors produce chemokines of two major groups: alpha and beta [61]. Evidence from murine models and human tumors propose that beta chemokines contribute vastly to macrophage and lymphocyte infiltration in tumors [75, 76]. A key molecular link between inflammation and tumor promotion and progression is transcription factor NF-kB, which regulates TNFα, interleukins, chemokines, and other molecular factors [77]. Although NF-kB is inactive in most cells, there is an activation state that is induced by a wide variety of inflammatory stimuli and carcinogens that, in turn, mediate tumorigenesis [78].

36.3.1 Relationship Between Depression and Inflammation

The relationship between the brain and the peripheral organs, often referred to as the "mind-body" connection, is based on alterations in the endocrine and immune systems that lead to the chemical changes that occur in clinical depression. Pro-inflammatory cytokines, particularly IL-6, have been found to occur in greater quantities in depressed patients [79]. It has also been shown that about 45 % of patients being treated medically with pro-inflammatory cytokine interferon-alpha (IFNα) developed symptoms of depression that reversed once the treatment ended [80]. Inflammation is not only a contributing factor in depression but also in many domains of medical illness. Among patients diagnosed with major depression, there is evidence to suggest that relationships exist between severity and duration of depression and increased prevalence of other disease processes, such as cardiovascular disease, type-2 diabetes, a variety of autoimmune diseases, and cancer [81]. Major depressive disorders (MDD) are also more prevalent in patients who suffer from illnesses that lead to chronic inflammation than healthy people [82]. While the presence

of an inflammatory disease may initiate depressive symptoms in patients without pre-existing psychological disorders, inflammation also occurs in depressed patients who are not suffering from concurrent inflammatory disorders [83].

The brain is influenced by the peripheral immune system where molecules such as cytokines, chemokines, and glucocorticoids originating in the peripheral organs can affect the neuronal pathways implicated in depression [79, 84]. Recently, it has been shown that symptoms of illness (fatigue, decreased appetite, social withdrawal, disturbed sleep cycles, anhedonia and mild cognitive impairment), and the normal bodily response to infection are triggered by pro-inflammatory cytokines, including IL-1α and β, TNFα, and IL-6 [79]. These cytokines are responsible for developing the body's inflammatory (local and systemic) response to invading microbes. In doing so, they also impact neural circuitry within the brain, resulting in the behavioral symptoms of illness. Such illness characteristics are remarkably similar to the symptoms of clinical depression. It is generally the role of anti-inflammatory cytokines to regulate the duration of these illness symptoms, possibly by inhibiting pro-inflammatory cytokine production and interfering with pro-inflammatory cytokine signaling [85].

Despite evidence to support the mechanisms by which pro-inflammatory cytokines act on the brain, the directionality of the inflammation–depression relationship is as yet unclear. As mentioned above, there is also research to suggest that depression may predispose people to developing illness. One study attempting to examine the directionality of the inflammation–depression relationship found that baseline depression scores of healthy (no medical illness) patients independently predicted change in IL-6. In contrast, IL-6 did not predict change in depression score [86]. The implication of those findings suggests that depression in previously healthy people may lead to inflammation and inflammation may be the mechanism through which depression potentiates chronic illness [87–89].

36.3.2 Relationship Between Obesity, Type-2 Diabetes, and Inflammation

The prevalence of obesity is increasing significantly in the U.S. and it is estimated that nearly two thirds of the population is overweight or obese [31]. Obesity is associated with a chronic, low-grade inflammation and can itself be viewed as an inflammatory condition since weight gain activates inflammatory pathways [90]. Serum levels of pro-inflammatory cytokines, including IL-6, TNFα, and C-reactive protein (CRP) are generally all elevated in individuals with obesity and insulin resistance [91].

Thus, the adipocyte is an active participant in the generation of the inflammatory state in obesity. Increased levels of cytokines lead to hepatic production and secretion of CRP, plasminogen activator inhibitor-1 (PAI-1), amyloid-A, alpha1-acid glycoprotein, and haptoglobin, which are all inflammatory markers that appear in the early stages of type-2 diabetes and increase as the disease progresses [92]. Obesity may stimulate inflammation through oxidative stress, which can result either from high levels of free radical production, a decrease in endogenous antioxidant defenses, or both [93]. The oxidative stress activates the pro-inflammatory transcription factor, NF-kB, continuing to promote low-grade chronic inflammation [94].

Several epidemiological studies have demonstrated that elevated weight and obesity, defined by a BMI higher than 25, result in significant increase for risk of cancer [95]. Park et al. examined how obesity enhanced cancer risk and development by studying HCC in mice and showed that dietary and genetic obesity promoted the growth of liver tumors [96]. There was a direct association between obesity-promoted HCC development and enhanced production of the tumor-promoting cytokines IL-6 and TNFα, both of which cause hepatic inflammation and activate the oncogenic transcription factor STAT3. The data suggest that inflammatory mechanisms may mediate the association between obesity and cancer development.

36.3.3 Inflammation and Treatment Considerations

Recent work has addressed whether psychological intervention can disrupt chronic inflammation and its resultant carcinogenic processes. A few promising studies have attempted to shed light on the answer by targeting depressive symptoms in patients diagnosed with cancer. Another approach has been the pharmacological treatment of depression, particularly with regard to selective serotonin-reuptake inhibitors (SSRIs) and tricyclics. Researchers have found that activation of the serotonin 5-hydroxytryptamine (5-HT) 2A receptor, known for its role in brain neurotransmission, results in inhibition of TNFα-mediated inflammation [97, 159]. One clinical trial that involved SSRI treatment of patients with major depression demonstrated a significant decrease in TNFα and CRP [98, 160]. CRP blood level has recently been shown to be an important and independent predictor of clinical HCC prognosis [99]. Other studies found that among patients with major depression treated with an SSRI, IL6, IL1β and TNFα levels were significantly lower post treatment and it has been demonstrated that the presence of serotonin is required for expression of the inflammatory markers IL-6 and TNFα [98, 100].

The interaction between psychological distress and chronic inflammation is of great interest. While the

directionality of this relationship remains unclear, and there is even evidence supporting bi-directionality, data suggest that psychological factors such as major depression, anxiety, chronic and daily life stress and anger suppression may trigger an inflammatory response. Unregulated, and often aggravated by the contribution of behavioral factors (dietary obesity, smoking, sedentary lifestyle), such inflammatory responses often develop into chronic disease. Thus, psychosocial factors are of critical importance, as they are often modifiable and such psychological interventions may alter or even prevent the course of chronic diseases associated with cancer development.

36.4 Evaluation of Psychiatric 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 [101], 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 36.1).

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 36.2 provides information regarding the number of items, scales, response scales, time frame, cut-off scores, and information regarding the reliability and validity of the instrument.

Table 36.3 provides questions that may facilitate the assessment of cancer-related symptoms 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 36.4 provides interview questions that may be useful in the evaluation of patients diagnosed with HCC.

The most challenging aspect of diagnosis of a psychiatric disorder in cancer patients is the differential diagnosis of psychiatric 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 appropriate diagnosis and treatment recommendations. Recommendations from a mental health professional may not result in a psychiatric diagnosis but further medical work-up to rule out medical or medication-related symptoms.

36.5 Psychosocial Distress

A significant proportion of patients who are diagnosed with cancer have some level of psychiatric distress [101]. Patients diagnosed with liver 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. HCC 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 [102, 103].

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 impairs 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 may 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 [104]. 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 [105–107]. Due to the risk factors associated with development of HCC (e.g., substance abuse), 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 [108]. In addition, family caregivers of patients with HCC 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 [109, 110]. Patients may also express regrets for prior behaviors that lead to the development of their cancer. It is also not uncommon for childhood and

Table 36.1 Instruments to assess psychological cancer-related symptoms in adults with HCC

Symptom	# of items and scales	Response items and time frame	Reliability		Validity		
			Temporal stability	Internal consistency	Content	Criterion	Construct
Depression							
Beck depression inventory (BDI-II) [1–3]	21 Summed Total score	0 = least severe/absent 3 = most severe *Past 2 weeks*	+**	+$^{\alpha\alpha}$	+	+	+
Center for epidemiological studies depression scale (CES-D) [4, 5]	20 Summed total score	0 = rarely/ none of the time 3 = most of all of the time *Past week*	+**	+$^{\alpha\alpha}$	+	+	+
The hospital anxiety and depression scale (HADS) [6–8]	14 1. Anxiety 2. Depression	0–3; multiple response sets	+**	+$^{\alpha\alpha}$		+	
Patient health questionnaire—depression scale (PHQ) [9–11]	9	0 = not at all 3 = nearly every day *Past 2 weeks*	+**	+$^{\alpha\alpha}$	+	+	+
Anxiety							
State trait anxiety inventory (STAI) [12–14]	40 1. State anxiety 2. Trait anxiety	1 = not at all/almost never 4 = very much so/almost always	+**/–	+$^{\alpha\alpha}$	+	+	+
The hospital anxiety and depression scale (HADS) [6–8]	14	0–3; multiple response sets	+**	+$^{\alpha\alpha}$		+	
Quality of life							
Functional adjustment to cancer therapy-hepatobiliary questionnaire (FACT-Hep) [15]	45 1. Social/family well being 2. Functional well being 3. Physical well being 4. Emotional well being 5. Symptoms and side effects	0 = not at all 4 = very much *Past week*	+**	+$^{\alpha\alpha}$	+	+	
EORTC quality of life questionnaire—hepatocellular carcinoma EORTC-QLQ-HCC [16, 17]	48 1. Functional • Physical • Role • Cognitive • Emotional • Social 2. Symptom • Fatigue • Pain • Nausea and vomiting 3. Global Health 4. Quality of Life	1 = not at all 4 = very much -or- 1 = very poor 7 = excellent *Past week*		+$^{\alpha}$	+	+	+

(continued)

Table 36.1 (continued)

Symptom	# of items and scales	Response items and time frame	Reliability		Validity		
			Temporal stability	Internal consistency	Content	Criterion	Construct
Medical outcome study 36-item short(SF-36) Form health survey [18–20]	36 1. Physical functioning (PF) 2. Role 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 limitations due to emotional problems (RE) 8. General mental health (MH)	Multiple response scales Higher scores indicating higher levels of functioning or well-being Standard: *4 weeks* Acute: *1 week*	+	+$^{\alpha\alpha}$	+	+	+
EuroQoL (EQ-5D) [21–24]	1. Descriptive index: • Mobility (MO) • Self-care (SC) • Usual activities (UA) • Pain/discomfort (PD) • Anxiety/depression (AD) 2. Visual analog scale	1 = no problems 2 = extreme problems -or- 100 = best imaginable health state 0 = worst imaginable health state	+	+$^{\alpha}$		+	+
Fatigue							
Revised piper fatigue scale (R-PFS) [25–28]	**22** 1. Behavioral/severity 2. Affective meaning 3. Sensory 4. Cognitive/mood	0 = no fatigue 10 = most severe fatigue *Present*	+	+$^{\alpha\alpha}$	+	+	+
Functional adjustment to cancer therapy—fatigue (FACT-F) [28, 29]	13 1. Social/family well being 2. Functional well being 3. Physical well being 4. Emotional well being 5. Fatigue subscale	0 = not at all 4 = very much *Past week*	+	+$^{\alpha\alpha}$		+	
Global fatigue index (GFI) [28, 30]	15 Degree, severity, distress and impact of fatigue	Scores range from 1 to 50 with higher scores indicating greater impairment from fatigue 100-mm visual analog, later changed to 10-point Likert		+$^{\alpha\alpha}$		+	+
Multidimensional fatigue inventory (MFI) [28, 31]	20 Phenomenology severity and impact of fatigue 1. General fatigue 2. Physical fatigue 3. Mental fatigue 4. Reduced motivation 5. Reduced activity	7-point Likert	+	+$^{\alpha\alpha}$		+	+

(continued)

Table 36.1 (continued)

Symptom	# of items and scales	Response items and time frame	Reliability		Validity		
			Temporal stability	Internal consistency	Content	Criterion	Construct
Schwartz cancer fatigue scale (SCFS) [28, 32]	28 Phenomenology and severity of fatigue 1. Physical 2. Emotional 3. Cognitive 4. Temporal	5-point Likert		+$^{\alpha\alpha}$	+		+
Fatigue questionnaire (FQ) [28, 33, 34]	11 1. Physical fatigue 2. Mental fatigue	Yes/No response -or- 4-point Likert		+$^{\alpha\alpha}$	+	+	
Fatigue symptom inventory (FSI) [28, 35, 36]	13 Intensity and duration of fatigue and its interference with quality of life	11-point Likert	–	+$^{\alpha\alpha}$			+
Sleep problems							
Pittsburgh sleep quality index (PSQI) [37]	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	4-point Likert Higher scores >5 indicate worse sleep quality	+*	+$^{\alpha\alpha}$	+	+	
Epworth sleepiness scale (ESS) [38–41]	8 Measures daytime sleepiness in adults	4-point Likert	+**	+$^{\alpha\alpha}$	+	+	
Pain							
Brief Pain Inventory (BPI) [42–44]	**23** 1. Pain intensity 2. Relief 3. Quality 4. Patients' perception of the cause of pain	11-point Likert *Past* 24 h	+**	+$^{\alpha\alpha}$	+		
Multidimensional pain inventory (MPI) [45]	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 3. Part III assesses patients' report of the frequency with which they engage in four categories of common everyday activities	7-point Likert	+*	+$^{\alpha}$	+	+	+

(continued)

Table 36.1 (continued)

Symptom	# of items and scales	Response items and time frame	Reliability		Validity		
			Temporal stability	Internal consistency	Content	Criterion	Construct
Visual analog scale (VAS) [44, 46, 47]	1 Sensory intensity affective magnitude of pain	100-mm visual analog scale 100 = worst pain imaginable 0 = no pain	+	+	+	+	+
Appetite/Cachexia							
Functional assessment of anorexia/cachexia therapy (FAACT) [48]	12 Anorexia/cachexia-related concerns	0 = not at all 4 = very much *Past week*		+$^{\alpha}$			+
Appetite and diet assessment tool (ADAT) [49, 50]	44 Appetite and change in appetite	5-point Likert scale *Acute and past week*					
Nausea and vomiting							
Nausea rating index (NRI) [51]	9 1. Sensory 2.Affective 3. Evaluative 4. Miscellaneous	Rank values		+$^{\alpha\alpha}$		+	
Overall nausea intensity (ONI) [51]	1	Rank values 0 = no nausea 5 = excruciating					
Cancer-related symptoms							
Memorial symptom assessment scale (MSAS) [52]	32 Severity, frequency, and distress of physical and psychological problems	2 or 3-point Likert scale		+$^{\alpha\alpha}$		+	
Multidimensional							
Four-Dimensional Symptom Questionnaire (4DSQ)	50 1. Distress 2. Depression 3. Anxiety 4. Somatization	Multiple response sets *Past week*		+$^{\alpha\alpha}$		+	
Distress thermometer [53]	1 Assess psychological distress	Self-report 11-point Likert scale 0 = no distress 10 = extreme distress				+	
Spiritual							
Functional assessment of chronic illness therapy-spiritual well-being subscale (FACIT-SP) [54]	12 1. Meaning/peace 2. Faith	0 = not at all 4 = very much *Past week*		+$^{\alpha\alpha}$	+	+	
Cognitive functioning							

(continued)

Table 36.1 (continued)

Symptom	# of items and scales	Response items and time frame	Reliability		Validity		
			Temporal stability	Internal consistency	Content	Criterion	Construct
Cognistat neurobehavioral cognitive status examination (Cognistat) [55–58]	1. Orientation 2. Attention 3. Comprehension 4. Repetition 5. Naming 6. Constructional praxis 7. Memory 8. Calculations 9. Similarities 10. Judgement	A score of 23 or lower is indicative of cognitive impairment	−			−	+
Mini mental status exam (MMSE) [59]	11 1. Orientation 2. Registration 3. Attention 4. Calculation 5. Recall 6. Language	Multiple responses *Acute*	+			+	
Substance use							
Fagerström test for nicotine depedence (FTND) [60]	6 Nicotine dependence	Multiple responses		−	+	+	
Alcohol use disorders identification test (AUDIT) [61]	10	3 or 5-point Likert scale *Past year*		$+^{\alpha}$		+	+

*$p \leq 0.05$
**$p \leq 0.001$
$^{\alpha}0.70 \leq \alpha \geq 0.80$
$^{\alpha\alpha}\alpha \geq 0.80$

Table 36.2 Interview questions to assess cancer-related symptoms

When did the symptom begin
Have there been any changes 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 worsen 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 are the symptoms (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 36.3 Interview questions for psychosocial assessment

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 the 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 prescribed for symptoms, adherence, response
Belief about symptoms and illness	Beliefs regarding symptoms Understanding of illness, severity, prognosis. 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 used 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 Frequency, duration, fluctuations in use, treatment and response if indicated
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 many strengths be used in treatment Which weakness/strengths interfere with treatment
Social resources (friends, family and school/work)	Support Family Friends Work/school Quantity and quality of support Support increase or decrease 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)
Logistic factors	Problems with transportation, finances, caregiving?

Table 36.4 Sleep hygiene practices 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 h 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 no less than 2 h before bedtime
Encouraging the patient to keep regular bedtime and awakening hours
Minimizing and coordinating necessary bedside contacts for inpatients
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

adolescent issues (e.g., abuse, neglect) that may have contributed to the onset of risk behaviors to surface at the time of diagnosis or over the course of treatment, as the patient may become increasingly vulnerable and dependent on health care professionals and family caregivers [111].

The popular press has exaggerated the benefits of "fighting spirit" and as a result, some patients diagnosed with cancer present to their health care 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 [112–146].

Benefit finding or posttraumatic growth (PTG) has gained increased attention in oncology 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" [115]. In hepatobiliary carcinoma, 50 % of patients report positive changes in their life after a diagnosis of cancer [116]. 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 [115, 117]. However, patients with hepatobiliary carcinoma reported a lower mean PTG score than breast cancer patients [116] 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 [115, 116, 118]. Patients diagnosed with HCC who reported higher levels of PTG were also found to have better immune system functioning [119]. Further research is warranted in understanding the construct (definition), process, and health outcomes associated with PTG.

36.6 Common Presenting Problems in HCC

36.6.1 Psychiatric Disorders and Treatment Recommendations

In addition to the emotional and psychiatric reactions to the diagnosis of cancer as described in the previous section, persistent psychiatric 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), 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 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, majority of the research referenced will be in regard to research that has been conducted in cancer patients more broadly.

36.6.2 Adjustment Disorder

Adjustment disorder is the most frequently diagnosed psychiatric 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)" [120]. 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 [120]. In a study of a mixed sample of cancer patients, 15 % of patients (49 % of all psychiatric diagnoses) met the DSM-5 criteria for Adjustment Disorder with depressed or anxious mood and Adjustment Disorder. 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 [104]. 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 MDD and Generalized Anxiety Disorder (GAD).

36.6.3 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 0–50 % of patients diagnosed with cancer may meet the DSM-5 criteria for MDD [121]. An additional 20 % may meet the criteria for depression spectrum disorders [121]. For a complete review, Massie [121] provides a summary of the prevalence rate according to cancer type, method of measurement, and timing of assessment.

MDD should be differentiated from an Adjustment Disorder with depression. In MDD, the number of symptoms required (5 or more) and the duration of these symptoms (2 weeks or longer for MDD) differs from Adjustment Disorder. Symptoms of MDD may include (1) depressed mood; (2) significant weight loss when not dieting or weight gain or decreased appetite; (3) insomnia or hypersomnia; (4) psychomotor agitation or psychomotor retardation; (5) feelings of worthlessness or excessive inappropriate guilt; (6) diminished ability to think or concentrate, or indecisiveness; (7) recurrent thoughts of death (not fear of dying), recurrent suicidal ideation without a specific plan, or suicide or attempt or specific plan for committing suicide; (8) fatigue or loss of energy; and (9) markedly diminished interest or pleasure in activities. These symptoms must occur most days, or nearly every day, as indicated by either subjective report or observation made by others [120].

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, 122–130]. 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 36.5.

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 [131, 132]. 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, 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.

Table 36.5 Cox regression analysis of sociodemographic, disease-specific variables, and depressive symptoms affecting survival ($N = 101$)

Variable	B (SE)	Wald	*p* level	95 % CI	
				Lower	Upper
Diagnosis					
HCC CCC NET METS	−0.001 (0.465) −0.021 (0.876) −0.575 (0.943)	0.488 0.001 0.001 0.371	0.92 0.99 0.98 0.54	0.402 0.176 0.280	2.491 4.454 11.286
Gender					
Male/Female	−0.417	1.355	0.24	0.752	3.066
Age					
<50 >50	−0.046 (1.131) −0.072 (1.115)	0.009 0.002 0.004	0.99 0.97 0.95	0.104 0.105	8.761 8.278
Ethnicity					
Caussian/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/hypol	−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* Cholangio carcinoma; *NET* Neuroendocrine carcinoma of the liver; *METS* Colorectal carcinoma with liver metastases; *HIA* Hepatic

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 [133, 134]. 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 five years, long-term antidepressant medication may be recommended [135, 136]. Psychosocial interventions are also being developed to reduce distress and depression in patients with cancer [137–140].

36.6.4 Anxiety Disorders and Phobias

Like depression, anxiety is also an important factor in cancer treatment, as these symptoms can affect adherence to medical treatments [141]. Studies have previously found that approximately 44 % of patients reported some level of anxiety and 23 % of patients reported significant anxiety that impaired functioning [142, 143]. 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 [120]. A person with GAD has difficulty controlling worry, and it is often associated with three or more of the following symptoms: (1) edginess or restlessness; (2) tiring easily or being more fatigued than usual; (3) impaired concentration or mind going blank; (4) irritability; (5) increased muscle tension or soreness; and (6) difficulties sleeping (e.g., trouble falling asleep, staying asleep, restlessness at night, or unsatisfying sleep). The anxiety must cause marked impairment in social, occupational, or educational functioning [120].

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 one 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 % [144, 145]. Limitations of previous research include the lack of assessing traumatic events and PTSD prior to the diagnosis of cancer. Veterans may be overrepresented in this population due to the risk factors associated with HCC and therefore are more likely to present with a current or past history of PTSD when compared to other cancer types [146–148].

In other cancer types, predictors of PTSD include dissociative symptoms, greater distress at the time of diagnosis, prior negative life stressors [149], a history of psychological problems, female gender [150], younger age at diagnosis [151, 152], lower socio-economic status [151], lower education [151, 153], avoidant coping style [154], low social support [154, 155], and reduced physical functioning [153]. Although the treatment of PTSD in other populations has been extensively reported [156–160], no study to our knowledge has tested the efficacy of interventions to treat PTSD in patients diagnosed with cancer.

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 [120]. The attacks may last a few minutes to hours and often come on suddenly. To meet the DSM-5 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 [120].

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 the prevalence of panic attacks was approximately 20 % in a sample of hospitalized cancer patients [161]. 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. The symptoms include: (1) palpitations, pounding heart, or accelerated heart rate; (2) sweating; (3) trembling or shaking; (4) sensations of shortness of breath or smothering; (5) feeling of choking; (6) chest pain or discomfort; (7) nausea or abdominal distress; (8) feeling dizzy, unsteady, lightheaded, or faint; (9) chills or heat sensations; (10) paresthesia (numbness or tingling sensations); (11) derealization (feelings of unreality) or depersonalization (being detached from oneself); (12) fear of losing control or going crazy; and (13) fear of dying. 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 [120]. 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 [120]. Research has been conducted in regard to stress-reducing medical devices (e.g., butterfly needles) which have been demonstrated to be effective in pediatric and adult patients undergoing chemotherapy [162].

36.6.5 Substance Abuse

The DSM-5 has redefined alcohol abuse according to symptoms and also the severity. The presence of at least two of the following symptoms indicates Alcohol Use Disorder (AUD) and the severity is defined by mild (presence of 2–3 symptoms), moderate (4–5 symptoms) or severe (presence of 6 more symptoms): (1) had times when you ended up drinking more or longer than intended; (2) more than once wanted to cut down or stop drinking but tried and could not; (3) spent a lot of time drinking or being sick or getting over the after effects; (4) wanted to drink so badly you could not think of anything else; (5) found that drinking or being sick from drinking often interfered with taking care of home or family or caused problems at school or work; (6) continued to drink even if it caused trouble with family or friends (7) given up or cut back on activities that were important or interesting to you or brave your pleasure in order to drink; (8) more than once got into situation while or after drinking

that increased your chances of getting hurt (e.g., driving, swimming, unsafe sex); (9) continued to drink even though it was making you feel depressed or anxious or adding to a health problem or after having had a memory blackout; (10) had to drink much more than you once did to get the effect you want or found that your usual number of drinks had much less effect than before; and (11) found that when the effects of alcohol were wearing off, you had withdrawal symptoms such as trouble sleeping, shakiness, or a seizure or sensed things were not there.

Whether the individual currently uses drugs or if they have a distant history of drug abuse, continued evaluation and treatment of substance use when indicated is imperative secondary to the high relapse rates observed in substance abuse [163, 164]. Persons who have a history of alcohol or drug abuse have the risk of relapse, particularly when facing major life stressors [165, 166]. All patients with chronic liver disease (CLD), not only those with alcohol-related HCC, should be evaluated for current drug and alcohol use, as alcohol and drugs have also been found to have a synergistic effect on the development of cirrhosis and may increase the rate of disease progression [167].

Identifying whether a patient is diagnosed with substance abuse is critical to the immediate care of the patient. If the patient has an active alcohol abuse disorder, assistance with addiction counseling may be essential to their initial stabilization and their ability to participate in treatment planning and adhere 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 [168], increased rates of pulmonary complications [28, 169–171], and infection [169, 170, 172]. Effective interventions for excessive alcohol consumption have been reported and tailored interventions have been effective prior to elective surgery [173].

Approximately 55 % of the general population has a lifetime history of tobacco use [120]. Tobacco use has been found to be high in patients diagnosed with HCC [174]. 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 [175], slowed wound healing [176, 177], pneumonia [171], poor outcomes after transplantation [29, 178], pulmonary complications [28, 179], 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 [180–184].

36.7 Cancer-Related Symptoms and Treatment Recommendations

The National Institutes of Health (NIH) consensus statement of 2002 concluded that the three most common and untreated cancer-related symptoms were pain, fatigue, and depression [185]. Approximately 40 % of patients at the time of diagnosis reported pain, 15 % reported weakness or malaise [174], and 37 % depressive symptoms [7, 174]. These cancer-related symptoms, as well as others, if left untreated can significantly impair a 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 [186]. Novel treatments are currently being tested to treat symptom clusters rather than each symptom independently [186]. 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 provide a brief overview of pharmacological and behavioral treatments recommendations.

36.7.1 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 National Cancer Institute (NCI), fatigue occurs in 14–96 % of people with cancer [187–190]. 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 [187, 191–197]. 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 [198–215]. Behavioral interventions such as improved nutrition as well as energy conservation and restoration activities may also be recommended [216–219].

36.7.2 Pain

Approximately 40 % of patients diagnosed with HCC report upper quadrant abdominal pain and/or pain in their shoulder at the time of diagnosis [174]. 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 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. Several standardized instruments that are useful in measuring pain in a clinical or research setting, including the most commonly employed measure of pain are mentioned in Table 36.1.

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 CLD is challenging for several reasons. A large percentage of patients may have a history of substance abuse making both patients and health providers reluctant to prescribe narcotics which are often the treatment of choice for cancer-related pain [220–224]. With increased regulation of narcotics, health care providers have become increasingly reluctant to prescribe narcotics and as a result patients' pain is often undertreated [225–231] 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 [232, 233]. It is recommended that medication 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 health care provider should be aware that patients will request higher doses over time, and that this should not 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 medication occasionally to decrease tolerance [234–237]. Multiple delivery systems (e.g., orally, as suppositories, and intravenously) have been developed to ensure effective management of pain 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 health care professionals involved in the pharmacological management of the pain as well as how a patient's prior history may affect patient or health care provider perceptions associated with pain management. 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 [238–242]. Relaxation techniques such as progressive muscle relaxation or autogenic are most often employed to treat pain [243–247]. Heat or cold packs are also used to decrease pain as well as massage, pressure, and vibration [248]. In some instances, exercise and/or frequent changes of position may be recommended based on the type of pain the patient presents [248]. If the pain persists, invasive treatments including nerve blocks and surgical interventions are available to patients.

36.7.3 Sleep Problems

While sleep disorders occur in 12–25 % of the general population it is estimated that 45 % of cancer patients experience sleep disturbance [197, 249–253]. In a sample of patients diagnosed with hepatobiliary carcinoma, 59 % of patients reported poor sleep quality; 43 % reported sleeping $\leq$6 h and 2 % $\geq$10 h [254]. After adjusting for factors known to contribute to survival, a curvilinear relationship was observed between sleep duration and mortality; short and long sleep durations were associated with increased mortality [254]. We observed that IL-2 was significantly associated with survival and when IL-2 was added to the model sleep duration, it was no longer significant demonstrating mediation using the Baron and Kenny mediational modeling [254].

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 [251, 255]. Medication including vitamins, corticosteroids, neuroleptics, stimulants,

Table 36.6 Meta-analysis of interventions for patients diagnosed with cancer

Outcome	Citation	Effect (95 % CI)	*N*	*p*-value
Depression (CBT)	Random combined	0.42 (−0.452–0.536)	66	0.663
		0.096 (−0.131–0.322)	303	0.405
		0.493 (−0.340–1.327)	26	0.212
		1.829 (1.324–2.335)	89	0.000
		3.424 (2.754–4.095)	89	0.000
		1.424 (0.767–2.081)	48	0.000
		1.206 (0.217–2.194)	621	0.017
Anxiety (CBT)	Random combined	3.060 (2.277–3.843)	59	0.000
		0.027 (−0.200–0.252)	303	0.817
		0.528 (−0.064–1.120)	48	0.069
		2.342 (1.573–3.110)	48	0.000
		3.516 (2.564–4.468)	48	0.000
		2.709 (1.887–3.531)	48	0.000
		1.999 (0.692–3.306)	554	0.003
Pain (Education)	Random combined	0.610 (0.110–1.110)	67	0.014
		0.242 (−0.380–0.565)	43	0.426
		0.232 (−0.373–0.837)	45	0.433
		0.060 (−0.274–0.395)	140	0.721
		0.243 (−0.005–0.490)	295	0.055

sedatives and hypnotics, anticonvulsants, and sympathomimetic may result in sleep disturbances [255]. 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 [255–257].

Evaluation and treatment of sleep disorders may include screening by a health care 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 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 metabolism of these drugs suggest that the patient should be started on a reduced dose [232, 233]. For any patient, the use of benzodiazepines for more than two weeks is not recommended due to psychological or physical dependence [250, 251]. However, the advantages and disadvantages of using sleep aids should be weighed as sleep deprivation may also have negative health and psychological consequences [256, 258, 259]. Behavioral interventions for insomnia include stimulus control and sleep hygiene techniques, [251, 260–266] relaxation techniques, [260] as well as cognitive-behavioral strategies to reduce anxiety or fears may also be effective in decreasing insomnia [260]. Table 36.6 provides recommendations from the NCI regarding sleep hygiene strategies specifically for patients diagnosed with cancer [261].

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 respiration. Obstructive sleep apnea is a result of the lack of air flow into or out of the person's nose or mouth [267, 268]. 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 HCC, 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.

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 is 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 [269].

36.7.4 Nausea and Vomiting

Nausea may be defined as an unpleasant wavelike feeling at the back of the throat or in the stomach that may involve the forceful elimination of the contents of the stomach [270]. For patients diagnosed with HCC, loss of appetite and nausea may be associated with the disease. In addition, several of the chemotherapy agents utilized to treat HCC (e.g., Cisplatin, Gemzar, Oxaliplatin) have varying levels of emetic effects [271–273]. 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, and electrolyte imbalance) [274, 275]. 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 health care providers but also intervene behaviorally to facilitate the response to the anti-emetics that are given prophylactically 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 [276–280]. Anticipatory nausea is the conditioned response of an odor, food, setting, or event in which the person experiences 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 [281–283]. In regard to the other types of nausea, behavioral treatments may facilitate the effectiveness of the anti-emetic medication but only the results of behavioral intervention have received mixed results in regard to their effectiveness with immediate, delayed, or persistent nausea and vomiting [284, 285].

36.7.5 Sexual Dysfunction

Sexual problems have been studied in patients with cancer of the reproductive organs and found to be higher than the general population [286–289]. Although little research has been conducted regarding sexual dysfunction in HCC, a recent study reported the prevalence of sexual dysfunction to be approximately 25 % [290]. Andersen et al. [291], 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. 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 medication); (5) cirrhosis; and (6) comorbid psychological symptoms (e.g., depression, anxiety) [290].

Zifroni et al. [292] reported that men with CLD and a Child-Pugh score of B or C reported higher rates of sexual dysfunction and significant reductions in free testosterone levels. In a recent study with patients diagnosed with HCC, no difference was found in those men who had Child's Pugh B or C scores and rates of sexual dysfunction when compared to those who had a Child's A score in both patients with HCC and CLD [290]. 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 [290]. The high rates of sexual dysfunction in HCC patients were found to be secondary to medical conditions and medications associated with increased sexual morbidity [290]. 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 [293]. 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 gonadotrophin stimulation; (4) in estradiol and luteinizing and follicle stimulating hormones levels; and (5) in binding capacities of sex steroid binding globulin [294–298].

Psychiatric symptoms such as anxiety, depression, and substance abuse 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 [299]. Furthermore, chronic alcohol use has been associated with male erectile dysfunction [300]. In the recent study of patients with HCC, individuals who reported a sexual problem and/or met the criteria for a DSM-5 diagnosis for a sexual disorder had a lower emotional well-being [301]. 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 are 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., dyspareunia, erectile dysfunction), excellent resources are available from the NCI and HRQL[302–304]. A comprehensive evaluation by a specialist (e.g. urologist, gynecologist) may be recommended to facilitate appropriate diagnosis and treatment.

36.7.6 Cognitive Impairment

Delirium may be defined as "a disorder of global cerebral dysfunction characterized by disordered awareness, attention, and cognition" [305]. Delirium may be transient and fluctuate over the course of the day and can be classified as hyperactive, hypoactive, or mixed [306, 307]. 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 [308–310]. The prevalence of delirium in patients with cancer ranges from 28 to 48 % [311–313] and approximately 90 % of patients will experience delirium hours before death [311, 314, 315]. 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 [316]. 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.

36.8 Special Issues

36.8.1 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 to 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 [109, 317–323].

An entire literature has been devoted to understanding and ameliorating caregiver stress across chronic diseases and specifically cancer [324, 325]. 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 [323, 326–328]. 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 [323, 327, 328]. As a result of the stress associated with caregiving, suppression of immune system functioning

[329–331] and increased risk of cardiovascular disease and mortality have been reported [332–334].

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 [335]. 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.

Health care 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 [336]. 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 health care professional may want to initiate these discussions with the caregiver if appropriate, individually and possibly together with the patient if appropriate.

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 [337, 338]. 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 [339, 340].

Anticipatory grief, which is similar to the grief loved one's 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 [341, 342]. 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 [343, 344]. 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 [345–347].

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 MDDs, substance abuse, 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 concerning interventions to evaluate and treat complicated bereavement in caregivers is warranted.

36.8.2 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 NCI have excellent resources that provide information regarding communication of the diagnosis of cancer to children and adolescents at different developmental stages [348, 349]. 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.

36.8.3 Cultural, Ethnic, and Religious Factors Affecting the Care of the Patient with HCC

As with all cancers, 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 [350, 351]. Culture and/or religious affiliation is important to recognize in regard to the role beliefs, attitudes, and behaviors these factors 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 [352, 353]. 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 that are important in the diagnosis and treatment of cancer [354].

36.8.4 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 health care providers later when the patient may experience cognitive impairment 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.

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 ones 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 [117, 355–361]. Some individuals have an increased sense of closeness to their belief in a "higher power" while others feel anger or resentment [355]. 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 health care providers to stop active treatment and essentially give up hope for a cure or controlling the tumor growth. Hospice care in the U.S. 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.

36.8.5 Alternative or Complementary Medicine in HCC

A high percentage of patients with CLD and HCC seek out alternative or complementary interventions to treat their disease. Although milk thistle is one of the only herbal supplements which is known for its benefits on liver functioning [362–366], no clinical trials in HCC have been published [366]. 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 or treatments; and (3) lack of available information regarding metabolism of the drugs in the liver, particularly the cirrhotic liver. Encouraging dialog 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.

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Bringing It All Together

37

Brian I. Carr

Contents

37.1 Prevention

This can only be rationally approached if the predisposing factors or causes are known. Fortunately, most causes of HCC are known (Chaps. 1, 2, 4, 16–19) and strategies are available for treating hepatitis B (HBV) and hepatitis C (HCV) and counseling for alcoholism and obesity, as well as for limiting aflatoxin B_1 contamination of foodstuffs.

For HBV, three phases of prevention can now be discerned—primary, secondary, and tertiary. Primary prevention can be accomplished by vaccination, usually in the neonatal period. Secondary prevention can be accomplished by treatment of chronic carriers with the new suppressive HBV drugs and curative HCV drugs, to prevent disease progression to either cirrhosis or HCC (with or without cirrhosis). Tertiary prevention seems to be recently obtainable by use of these new therapies in carriers who have had resection and are at risk for HCC recurrence. New evidence is suggesting that such therapies can lower the recurrence rates. If this is supported by further and prospective clinical trials, then hepatitis therapy will need to be also considered as part of cancer therapy. For HCV, the new curative drugs seem capable of being effective in secondary and possibly in tertiary prevention, though primary prevention vaccines are not yet available.

The storage of village rice and peanuts in refrigerated granaries is likely to be helpful in suppressing growth of the carcinogenic fungi that contaminate stored grains in tropical and humid environments found in the third world. The mechanisms by which these risk factors might mediate HCC development (carcinogenesis) are discussed in Chaps. 2–4, 11, 13, 15, 18, and 19.

37.2 Surveillance

A primary purpose of surveillance is to diagnose the tumor at an early stage when there is the possibility of curative therapy (Chaps. 22 and 23). Much has been written on the subject of screening for HCC, including the usefulness of alpha-fetoprotein as a marker and the most reliable, simplest, and cheapest radiologic modality, especially in developing countries. 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 USA that has chronic HBV or 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 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 ultrasound scans and alpha-fetoprotein measurements, even though the latter are elevated in only 50 % of HCCs and there is no clear linearity between tumor size and alpha-fetoprotein

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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 who are diagnosed at advanced tumor stage. An ongoing conundrum is the fairly large number of patients who have diagnosis at advanced tumor stage, with or without prior screening.

37.3 Current Therapy

The treatment decisions for HCC depend on A. The extent of the tumor size, site of the tumor with respect to major vessels within the liver, number of tumor nodules, and the presence and extent of portal vein thrombosis/invasion (PVT); and B, the degree of associated liver fibrosis and damage (Fig. 37.1).

- Liver resection is a primary treatment option for patients without cirrhosis and for those with mild degrees of cirrhosis (Child-Pugh A), patients without portal hypertension and with normal plasma bilirubin levels. More recently, several studies have shown its safety and effectiveness even in the presence of branch PVT and well-preserved liver function (Chap. 31). More recently, laparoscopic liver resection, radiofrequency ablation, or microwave ablation have gained favor (Chaps. 30 and 31). The high recurrence rates in the years after resection have not been decreased by adjuvant chemotherapies, so far. However, recent reports using the more potent and effective anti-HBV (and likely HCV) therapies, suggest that in carriers of those diseases, antiviral therapy may turn out to be important in decreasing HCC recurrence in the postresection/ablation period. TACE, ^{90}Yttrium, or Sorafenib (failed STORM trial) have not thus far been shown to be useful in the adjuvant setting.
- Liver transplant is offered for patients who are not suitable for cure by resection, usually based on their liver

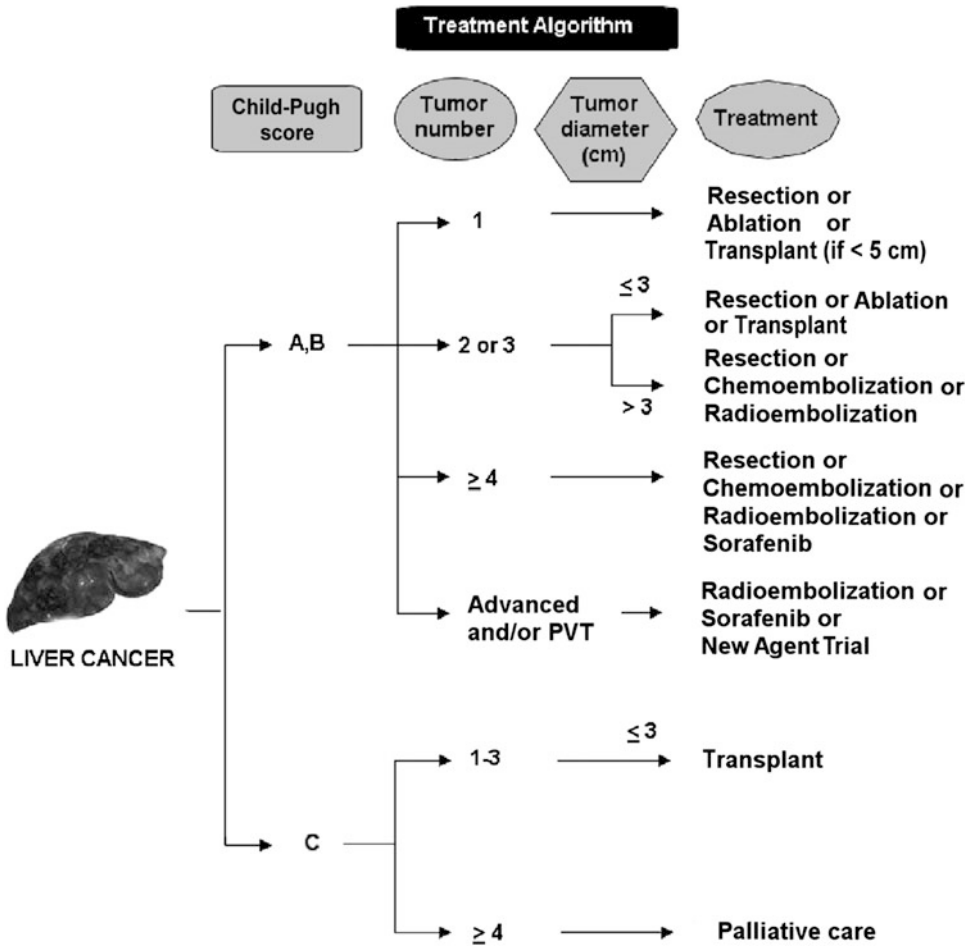

Fig. 37.1 Treatment decision algorithm. From figure 2 of Kokudo et al. [4], Copyright license approval # 3744900665218 obtained from John Wiley and Sons. *Child-Pugh score*, reflects the degree of liver damage

reserve, and who have tumors within the benchmark Milan criteria (one tumor <5 cm diameter or a maximum of three tumors, each with maximum diameter ≤ 3 cm. This is the preferred treatment in presence of Child-Pugh grade B cirrhosis. However, extended (UCSF) criteria are offered in increasing numbers of centers (Chap. 32). Increasingly, transplantation is being considered in selected patients with tumors that initially presented beyond the usual transplant criteria, but which are then successfully downstaged after local regional therapy.

Liver resection, transplantation, or tumor ablation (PEI, RFA, MWA) represent the only current therapies with potential for cure (Chaps. 29–32). Most HCC patients, however, are not candidates for these therapies at the time of diagnosis because of portal hypertension, poor liver function, tumor multifocality, PVT, and/or comorbidities (most often due to advanced tumor stage at diagnosis).

- Chemoembolization or TACE (transarterial chemoembolization) is the most commonly used nonsurgical treatment modality for these patients who cannot be offered 'curative' therapies (Chap. 33), although ^{90}Yttrium spheres regional therapy is increasingly popular (Chap. 35). However, there are not yet direct comparisons between TACE and ^{90}Yttrium spheres to guide choice of therapy modality. TACE is the standard of care for patients with multiple lesions and well-preserved liver function, with or without branch PVT and absence of metastasis. There is no tumor size limitation. Doxorubicin or cisplatin are well-studied, tolerated, and partially effective chemotherapy agents in this setting. A bilirubin of <2 mg/ml and absence of ascites or minimal ascites seem to offer the safest conditions. Chemotherapy is often mixed in an emulsion with Lipiodol (Ethiodol) and commonly used embolization materials include gelatin sponge particles (Gelfoam) or defined-size microspheres (Embosphere Microspheres). Repeat treatments are typically given every 2–4 months, depending on blood counts, liver function tests, and tumor size response and vascular responses on follow-up CAT scans. TACE in association with RFA has been reported to yield better outcomes than RFA alone. Tumor growth in a previously treated area of the liver is considered a treatment failure and cause for change of therapy. Typically, the choices are then ^{90}Yttrium spheres regional therapy (radioembolization with either Theraspheres or Sirspheres) or Sorafenib (Chaps. 33–35). Single large tumors in Child A or B cirrhosis that are unresectable and beyond transplant criteria can be treated with TACE or ^{90}Yttrium spheres.
- TACE needs to be used with caution in the presence of major branch PVT and not at all in presence of main stem PVT. By contrast, several papers have recently shown the relative safety of ^{90}Yttrium spheres in the presence of PVT. This is likely to make ^{90}Yttrium spheres, or Sorafenib, a first choice of therapy in presence of major branch PVT. For patients with tumor progression following TACE, the choice is ^{90}Yttrium spheres or oral Sorafenib therapy, since there is no trial data to indicate which is superior after TACE failure. For patients failing first-line ^{90}Yttrium spheres therapy, the second-line choice is Sorafenib.

  Recently, external beam radiation therapy (EBRT) has been offered to patients who are surgically unresectable and cannot have other local therapy, such as major branch PVT, with encouraging response and safety data. However, this is in early phases of evaluation.
- Patients failing TACE or ^{90}Yttrium spheres therapy or Sorafenib and who have good performance status, or who have metastasis, are typically offered clinical trial enrollment with new agents, if their performance status is satisfactory. Similarly, if they would otherwise have been offered TACE or ^{90}Yttrium spheres therapy or Sorafenib, but a clinical trial comparing any of those with a new agent is available, then enrollment in the trial may be reasonable, since none of those three modalities is curative in this setting.

37.4 The Multidisciplinary Team

It follows from the need for multiple specialists to be involved in diagnosis (primary care physician, hepatologist, radiologist, pathologist), in therapy (liver surgeon, liver transplant surgeon, radiologist, interventional radiologist, radiotherapist/nuclear medicine physician, amongst others), and in daily patient management (nurse coordinators, social worker, clinical psychologist) that comprehensive decision-making at both initial patient evaluation and as new treatments are needed, require a multidisciplinary team, that functions best when representatives of each of these specialties are together in the same room, evaluating not only the total patient physical and laboratory test results, radiology and pathology results, but also the patient home setting, upon which each patient depends for coping with the plethora of tests, appointments, and treatment toxicities, in addition to the heavy emotional burden on both patient and family. Optimal decision-making for total disease management and patient care thus requires a whole team, as well as an identifiable single physical or surgeon to integrate the results of all the tests across specialities, and who guides the patient through the complex medical maze of tests and procedures and in decision-making at each step. A 'contact'

nurse or coordinator, who is present at these discussions and who is accessible to the patient, is normally of profound importance in getting the patient through this medical/surgical maze. It is increasingly clear that patients are often in a state of anxiety at the beginning of the treatment evaluation process and may or may not hear or remember what team members advise. Therefore, a family member/friend is also extremely important for accompanying the patient at both Consultations and clinic follow-up appointments.

37.5 Some New Concepts and Controversies

A. **The role of tumor biopsy**. In solid tumor oncology, biopsy of the tumor is essential for proof of diagnosis and thus a prerequisite for therapy. In recent years, this has not been used by many hepatologists, though still used by oncologists, since, when there is a combination of a vascular lesion in a cirrhotic liver with elevated blood alpha-fetoprotein, then the diagnosis of HCC has such high probability in these circumstances (see Table 37.1, diagnosis). However, two recent circumstances oblige a reconsideration of biopsy. First, the new targeted therapies often are expected to work in the presence of their target in the tumor (e.g., Met inhibitors in the presence of Met protein, Chap. 34). Second, the new transcriptomics are providing molecular signatures for prognostication. In this regard, the underlying, non-HCC liver seems also to be particularly important (Chaps. 5–8 and 12). Thus, both tumor biopsy and liver biopsy seem to be increasingly important in the molecular medicine of HCC. However, the increasing ability to enrich for and thus sample circulating tumor cells and free tumor DNA in the circulation, may further enhance the ease of "liquid biopsy," as well as offering a safe prospect for sequential tumor sampling during the changing course of an individual patient's tumor (Chaps. 5, 7, and 12), as well as the possibility for examining tumor heterogeneity within an individual's tumor nodule (Chap. 14). As liquid biopsy becomes standardized, it will offer the possibility of regular molecular sampling of an individual patient's tumor as it evolves during the course of the disease.

   It is our practice always to do a biopsy before treatment, whenever practical. We believe that this is important, since it gives us 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 is an increasing number of tests that are starting to permit us prognostic subgroup 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 1300 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, the even rarer risk of tumor bleed or other rarer complications associated with the presence of ascites, or the risk of not treating an HCC because a benign lesion is thought to be present.

B. **Molecular HCC classification**. The increasing evidence that patterns of gene expression are capable of predicting HCC biology (recurrence probability post resection, general prognosis or sensitivity to specific targeted therapies (Chaps. 5–8, 12, and 34) is likely sooner rather than later to become part of new HCC evaluation. Although right now these are research tools, ongoing clinical trials are attempting to validate them for use in clinical practice.

C. **The biobank**. In light of the above findings, it is becoming clearer that the routine development and use of tumor tissue, nontumor liver tissue, and blood biobanking will be needed to be incorporated into clinical practice, as specific patterns of molecular signatures come to be associated with prognostic use and the requirements for rational selection of therapeutic agents that work on specific cellular targets.

D. **Significance of tumor stability and responses to medical therapy**. Conventional clinical oncological practice uses changes in tumor size by CT or MRI scan, to measure response to chemotherapy or radiotherapy (including TACE and ^{90}Yttrium microspheres). However, it has become recently clear that changes in vascular intensity without decreased tumor size ('vascular response') are also a valid index of HCC response to therapy, and are thus acknowledged as part of the mRECIST response criteria. However, quantitative radiological means of assessing HCC vascular changes are still in need of standardization. In addition, stable tumor (unclear whether vascular response is required in this context), may also be clinically very useful for enhanced survival, as the SHARP phase III trial of Sorafenib versus placebo showed only 2 % radiological

Table 37.1 Summary of recommendations for HCC prevention, diagnosis, and therapies

Prevention
1. Hepatitis B vaccination is recommended for all newborns and for high-risk individuals (HBsAg-negative and anti-HBs-negative)
2. General preventive measures include the following: prevention of HBV/HCV transmission, avoidance of alcohol abuse, and control of metabolic disorders such as obesity and diabetes
3. Antiviral therapy as secondary prevention of HCC should follow guidelines for the management of chronic hepatitis B/C
4. Antiviral therapy should be considered after curative treatment for chronic viral hepatitis-related HCC in order to reduce the risk of recurrence
Diagnosis
1. HCC is diagnosed on the basis of either pathology or clinical criteria in case of the high-risk group (HBV/HCV positive or cirrhosis)
2. When HCC is suspected during surveillance in a high-risk patient, dynamic contrast-enhanced CT/MRI should be performed for diagnosis
3. In the high-risk group, HCC can be diagnosed for nodules ≥ 1 cm in diameter if one or two of the above-mentioned imaging techniques show typical features of HCC (for the diagnosis of nodules 1–2 cm in diameter, two or more imaging modalities are required if a suboptimal imaging technique is used). Typical features of HCC include arterial phase enhancement with washout in the portal or delayed phase
4. Nodules <1 cm in diameter can be diagnosed as HCC in high-risk patients when all of the following conditions are met: typical features of HCC in two or more of the above-mentioned imaging modalities and continuously rising serum alpha fetoprotein with hepatitis activity under control (C1)
5. Pathological diagnosis should be considered when the clinical criteria are not met or typical features of HCC are not present. The presence of indeterminate nodules despite imaging workup or pathologic examination needs to be followed up with repeated imaging and serum tumor marker analysis. Limitation of radiation exposure in diagnosis and staging is not considered relevant in patients with HCC. CT is essential for diagnosis and follow-up in HCC patients This guideline adopts the modified Union for International Cancer Control stages as a primary staging system, with the Barcelona Clinic Liver Cancer staging system serving as a complementary system
Treatment
1. Surgical resection is the first-line treatment for patients with intrahepatic single-nodular HCC and well-preserved liver function classified as Child-Pugh class A, without portal hypertension or hyperbilirubinemia. Limited resection can be selectively applied to HCC patients with liver function of Child-Pugh class A or superb B and with mild portal hypertension or mild hyperbilirubinemia. HCC resection can be considered in patients with three or fewer intrahepatic tumors without macrovascular portal or hepatic vein invasion, if hepatic function is well preserved. Laparoscopic resection can be considered for HCC located in the lateral section of the left lobe or in the anterolateral segment of the right lobe
2. RFA provides survival comparable to that of resection in patients with single-nodular HCCs ≤ 3 cm in diameter. RFA is superior to PEI in terms of anticancer effect and survival. For HCCs ≤ 2 cm in diameter, PEI can be considered if RFA is unfeasible, because the outcomes of both modalities are similar. Survival outcome can be improved by combining TACE and RFA compared to RFA alone in patients with tumors 3–5 cm in diameter if resection is unfeasible
3. Deceased donor liver transplantation is the first-line treatment for patients with single-nodular HCC <5 cm in diameter or three or fewer nodules ≤ 3 cm in diameter (Milan criteria), which are not suitable for resection. Locoregional therapies (local ablation or TACE) are recommended if the timing of transplantation is not predictable (bridge to transplant). Downstaging (e.g., with TACE) can be considered for HCCs exceeding the criteria for transplantation. Living donor liver transplantation is an effective alternative to deceased donor transplantation. An expanded indication for transplantation beyond the Milan criteria can be considered in HCC cases without clear vascular invasion or extrahepatic spread if other effective treatment options are inapplicable. Salvage transplantation can be indicated for recurrent HCC after resection according to the same criteria as for first-line transplantation
4. TACE or ^{90}Yttrium microspheres therapy is recommended for patients with good performance status without major vascular invasion or extrahepatic spread who are ineligible for surgical resection, liver transplantation, RFA, or PEI. TACE should be performed through tumor-feeding vessels using selective/superselective techniques to maximize antitumor activity and minimize hepatic damage
5. In case of portal vein invasion, TACE can be considered for patients with localized tumor and well-preserved liver function, but ^{90}Yttrium microspheres appear safer, probably with comparable efficacy. External beam radiation therapy (EBRT) can be considered for HCC patients with portal macroscopic vein invasion. So can sorafenib
6. Sorafenib is indicated for HCC patients with very well-preserved liver function (Child-Pugh class A and excellent B), good performance status, and regional lymph node or extrahepatic spread or for patients with tumor progression on other therapies
7. Cytotoxic chemotherapy can be considered for HCC patients with advanced tumors who have with well-preserved liver function and good performance status, in whom sorafenib therapy has failed. Adjuvant TACE, sorafenib, or cytotoxic chemotherapy are not recommended for HCC patients treated with curative resection, as there is no evidence for their adjuvant benefit, at the time of writing
8. Preemptive antiviral therapy is recommended for HBV carriers undergoing any cytotoxic chemotherapy to prevent reactivation

Adapted from Table 2, summary of the recommendations of the 2014 KLCSG-NCC Korea practice guidelines for the management of hepatocellular carcinoma. Korean Liver Cancer Study Group (KLCSG) et al. [5]

responses in the Sorafenib arm, yet patients in this arm had significantly enhanced survival.

E. **Advances in nonsurgical therapies**. There is a huge cornucopia of new agents (drugs, antibodies, chemical inhibitors) that target various steps in growth-related cellular pathways, most of which are in current clinical trials as either first-line (in comparison to sorafenib), or second-line therapy (after failure of sorafenib or other therapies such as TACE or ^{90}Yttrium microspheres). As sorafenib resistance is increasingly recognized, many newer clinical trials will follow the path of the last many years of chemotherapy and will be combined, especially those targeting parallel growth pathways or agents with nonoverlapping toxicities. In addition, a large number of newer agents are being developed and clinically tested that do not target specific growth pathways, such as modulators of tumor stem cells, differentiation-inducing drugs, or newer immune modulators (much current excitement over the possibilities of the PD-1 immune checkpoint modulators), such as Nivolumab (Opdivo®) or Pembrolizumab (Keytruda®). Many new clinical trials are also evaluating the combination of the new targeted therapies in conjunction with the older cytocidal therapies (TACE or ^{90}Yttrium microspheres).

F. **Inflammation and microenvironment**. The recent appreciation of the Glasgow index (albumin and C-reactive protein) as an independent risk factor in the prognosis of many tumors, including HCC (Chap. 15), has drawn attention to the non-HCC factors in tumor biology and prognosis (Chaps. 5, 6, and 11), in addition to the gene expression profiles mentioned in sections A and B, above. It is also becoming increasingly clear that many factors in the tumor environment (Chap. 11), both cellular (immune system, Chap. 13), as well as vascular endothelial cells and the noncellular components of the microenvironment, such as growth factors and inflammatory cytokines, and extracellular matrix proteins, all influence HCC biology, including sensitivity to Sorafenib (3). Seemingly also does the microbiota (Chap. 9). A future edition of this book will likely include this topic of microenvironment in depth.

G. **HCC prevention and early diagnosis**. Perhaps the two most important factors to influence prognosis are (1) the new availability of approved and potent hepatitis B and C inhibitors and (2) the fact that most HCCs are still diagnosed too late for "curative" therapies. Prevention and aggressive early detection strategies are clearly key to the latter. Prevention includes hepatitis vaccination (HBV) or therapy (HBV and HCV), clean water supplies in rural Asia and Africa (Aflatoxin B_1 contamination), refrigerated granaries in Asia and Africa (carcinogenic mycotoxins including Aflatoxin B_1), alcohol consumption counseling and therapy, and a massive obesity-prevention program (NASH, Chaps. 1, 2, 18, and 19) will be important in combating rich-world trends.

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