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Prevention of hepatocellular carcinoma: potential targets, experimental models, and clinical challenges

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Abstract

Chronic fibrotic liver diseases such as viral hepatitis eventually develop liver cirrhosis, which causes occurrence of hepatocellular carcinoma (HCC). Given the limited therapeutic efficacy in advanced HCC, prevention of HCC development could be an effective strategy for improving patient prognosis. However, there is still no established therapy to meet the goal. Studies have elucidated a wide variety of molecular mechanisms and signaling pathways involved in HCC development. Genetically-engineered or chemically-treated experimental models of cirrhosis and HCC have been developed and shown their potential value in investigating molecular therapeutic targets and diagnostic biomarkers for HCC prevention. In this review, we overview potential targets of prevention and currently available experimental models, and discuss strategies to translate the findings into clinical practice.

Keywords

Animal model; chemoprevention; clinical trial; hepatocellular carcinoma; liver cirrhosis; prevention

INTRODUCTION

Liver cancer, predominantly HCC, is the second and sixth most lethal cancer in men and women, respectively, and occurred in about 750,000 new patients and caused 700,000 death in 2008 [1]. More than 80% of the cases occur in developing countries in the Asia-Pacific regions and sub-Saharan Africa, and China alone accounts for more than 50% [2]. In the US, HCC is the most rapidly increasing cause of cancer-related mortality, and is ranked as third in men ages 40 to 60, clearly indicating its large socioeconomic impact [2,3]. The incidence of HCC tripled between 1975 and 2005 [4], and is assumed to increase in the next few years and remain high for the next two decades [5,6].

HCC occurs in liver affected with chronic predisposing conditions such as liver cirrhosis, which is seen in about 80% of HCC patients [7,8]. It is estimated that roughly 1~2% of population is affected with liver cirrhosis, and the risk of HCC increases according to

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CONFLICT OF INTEREST
Declared none.

severity of liver fibrosis and dysfunction [8-11]. Regular tumor surveillance for cirrhotic patients has enabled efficient early detection of HCC tumors to be eligible for potentially curative treatments: surgical resection, local ablation, and liver transplantation [12]. However, complete tumor removal is not equivalent to a cure because of persisting cirrhosis in remnant liver. In fact, repeated tumor recurrences are observed in nearly 80% of the patients, limiting improvement of 5-year survival up to 70-80% [7]. Once the tumor gets into advanced stage, there is no curative treatment option; even the recently introduced multi-kinase inhibitor, sorafenib, could yield survival benefit only up to several months [13,14].

The refractory nature of HCC suggests that prevention of its development in high-risk patients is the most effective strategy to substantially improve the mortality [15]. In addition, the high prevalence of liver cirrhosis has further highlighted the impact of HCC prevention on global health [16]. In fact, HCC is a more attractive target of preventive intervention compared to other cancer types such as prostate cancer [17], breast cancer [18], and keratinocyte carcinoma [19] because it is easier to identify individuals at risk (i.e., cancer-susceptible cohort) and to monitor treatment response due to the high incidence rate.

RISK FACTORS AND MOLECULAR MECHANISMS OF HCC DEVELOPMENT

A wide range of etiological agents contributes to hepatocarcinogenesis through specific molecular mechanisms, clearly indicating the necessity to develop strategies of HCC prevention accordingly.

Hepatitis B virus

Chronic infection of hepatitis B virus (HBV) is the most common global cause of HCC, affecting more than 350 million individuals (6% of world population) and being the dominant etiology especially in China and Africa [20]. HBV proteins as well as HBV DNA integration into host genome have been suggested to possess direct carcinogenic effect by activating *cis* and *trans* oncogenic signals in host hepatocytes [21]. In consistent, high serum HBV DNA level, indicative of increased viral replication, is predictive of HCC development, which is not necessarily accompanied with advanced liver fibrosis [22]. It has been suggested that certain HBV strains, e.g., genotype C in Asian population and genotype F in Alaskan population [23,24] or mutations in HBV genome, e.g., within precore and basal core promoter regions [23] increase the risk of HCC. Superinfection with hepatitis delta virus (HDV) is associated with severer hepatitis, accelerated fibrosis progression, and increased risk of HCC [25].

Hepatitis C virus

Hepatitis C virus (HCV) affects 170 million individuals worldwide, has been the major risk factor of HCC in many industrialized countries, and is contributing to the increasing HCC incidence in the US [4,26]. It is estimated that more than one million patients could develop HCV-related cirrhosis, hepatic decompensation, or HCC by 2020 in the US [27]. Clinically, incidence of HCV-related HCC increases according to severity of liver fibrosis, and in contrast to HBV-related HCC, patients with minimal fibrosis rarely develop HCC, suggesting that cirrhotic microenvironment is the major driver [8,11,28]. There are conflicting evidences that HCV viral factors such as serum RNA level increase HCC risk [29,30]. Some studies suggested that genotype 1 is associated with increased risk of HCC, although it may simply reflect that this genotype is refractory to antiviral therapies and has more chance to progress into advanced disease.

Exposure to environmental carcinogens

Dietary contamination with the potent hepatocarcinogen, aflatoxin B_1 (AFB_1, a natural mycotoxin produced by *Aspergillus* fungi) is observed in some (sub)tropical regions with warm and humid climate especially in Eastern and Southeastern Asia and sub-Saharan Africa, where HBV infection is prevalent [20]. AFB_1 causes a loss-of-function mutation in the "hotspot" codon 249 (G to T transversion) of exon 7 in the TP53 tumor suppressor gene [31], and exhibits remarkable synergistic hepatocarcinogenic effect with HBV [32,33]. Food or water contamination with other fungal toxins such as fumonisin, blue-green algae-derived microcystins, nitrosamine, and inorganic arsenic, and betel quid chewing are also suggested to increase HCC risk [20,34,35].

Alcohol

Liver cirrhosis induced by long-term, excessive intake of alcohol is a well-established risk factor of HCC, and is one of the major causes in North America and Western Europe [20,36]. It is likely that alcohol has an indirect carcinogenic effect through establishment of cirrhosis [36]. Heavy alcohol consumption shows synergism with HCV and possibly with HBV infection in promoting HCC development supposedly by accelerating cirrhosis progression.

Non-alcoholic fatty liver disease, obesity, and type 2 diabetes

Recent studies have suggested that many HCC cases developed in so-called cryptogenic cirrhosis may be attributable to non-alcoholic fatty liver disease (NAFLD), including non-alcoholic steatohepatitis (NASH) [37]. Epidemiological data have suggested tight association of NAFLD/NASH with visceral obesity and (mainly type 2) diabetes accompanied with insulin resistance, which were also independently reported as risk factors of HCC development [38,39].

Hepatic iron overload disorders: hemochromatosis

Genetic hemochromatosis (GH) is mostly caused by homozygous mutations in the hemochromatosis (*HFE*) gene, especially C282Y (more common in Caucasian) and to lesser extent H63D, which abnormally increase iron absorption and accumulation especially in the liver, heart, and pancreas. Established cirrhosis is an HCC risk factor in GH. A Swedish population-based cohort study reported an increased HCC incidence especially in male GH patients, mostly harboring the C282Y mutation [40].

In patients with chronic hepatitis C and advanced fibrosis, positive association between liver iron deposition and higher incidence of HCC and poor prognosis was observed [41]. Hepatic iron overload was associated with elevated levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), which signifies hepatic oxidative DNA damage in patients with chronic hepatitis C [42]. With an excess iron diet, transgenic mice expressing HCV polyprotein showed development of hepatic steatosis, ultrastructural alterations of mitochondria, and HCC accompanied with elevated levels of hepatic 8-OHdG [43].

Genetic risk factors

Recent studies have identified etiology-specific or independent host genetic polymorphisms associated with increased risk of HCC in patients with chronic liver diseases. A single nucleotide polymorphism (SNP) in epidermal growth factor (EGF) gene (SNP rs4444903) was associated with increased EGF expression and elevated risk of HCC development in cirrhotic patients [44,45]. Genome-wide association study (GWAS) have revealed a SNP (rs 17401966) possibly associated with altered expression and function of several potential tumor suppressor genes in 1p36.22 namely *KIF1B, UBE4B*, and *PGD* in HBV-related

Chinese HCC patients [46] and SNPs in *MICA* (rs rs2596542) [47] and *DEPDC5* (rs1012068) [48] in HCV-related Japanese HCC patients. These findings await future validation. Given the magnitude of risk for the loci are generally low (odds ratio well below 1.5), it may be more clinically meaningful to measure them simultaneously to evaluate their collective effect.

Other risk factors

HCC risk in primary biliary cirrhosis (PBC) with stage 4 cirrhosis is about the same as in HCV-related cirrhosis [49]. HCC occurs less frequently in autoimmune hepatitis (AIH) only after establishment of cirrhosis, suggesting that inflammation alone is not sufficient [50]. Only scarce epidemiological evidence exists for other rare forms of chronic liver diseases such as alpha 1-antitrypsin deficiency [51]. There are conflicting evidences regarding association of smoking with HCC risk, for which confounding effect of alcohol abuse cannot be fully excluded [2,20,52]. Human immunodeficiency virus (HIV) co-infection increases risk of HCC in patients with chronic viral hepatitis [53].

Heterogeneous distribution of HCC risk factors

There is a large disparity in the distribution of the risk factors and patient demographics across geographic sites; the majority of cases in developing countries in Eastern Asia and Africa are caused by HBV with or without exposure to AFB_1, whereas HCV-related HCC is more frequent in developed countries in the West and Japan [2]. In addition, in developing countries, the patients do not visit clinics until tumors get into advanced stage because early-stage HCC is generally asymptomatic. By contrast, in developed countries, HCC is diagnosed at earlier stage under the regular tumor surveillance program, and more likely to be treated with the curative therapies [7]. Thus, the strategy of patient management should be designed with specific consideration to the dominant etiologies, stage of disease, and available medical and socioeconomic resources available at each site. HBV and HCV contribute to development of more than 80% of HCC worldwide [1].

Altered molecular pathways in cirrhosis

Irrespective of the etiology, established cirrhosis serves as a milieu/microenvironment that fosters initiation and promotion of carcinogenesis by facilitating genetic aberrations and cellular transformation, which is often referred to as “field cancerization” or “field effect” [54-57]. For the majority of etiological agents, hepatocarcinogenesis is tightly associated with repeated cycles of hepatocyte death followed by regeneration and assumedly with accumulating potentially oncogenic mutations. This process could take several decades (typically 20-40 years) before establishment of liver cirrhosis, which may reflect the organ's resistance to replication errors [58]. Severity of liver fibrosis is correlated with increasing risk of HCC especially in HCV-infected patients [8,11].

Oxidative stress and inflammatory response are considered to play a central role in fibrosis progression and HCC development in NAFLD [59] and alcoholic hepatitis [60]. More frequent epigenomic alteration, impaired immune surveillance, and increased genetic susceptibility were suggested in alcoholic cirrhosis. Viral products such as HCV core protein may have direct carcinogenic effect by inducing generation of reactive oxygen species and transactivation of intracellular signaling cascades such as mitogen-activated protein kinase (MAPK) and activator protein (AP)-1 pathways [61].

Hepatocyte proliferation is generally decreased at the stage of cirrhosis after many rounds of regeneration accompanied with telomere shortening that induces chromosomal instability, impaired hepatocyte proliferation, and abnormality in cellular senescence [2]. Several molecular dysregulations observed in HCC tumors could be involved in

hepatocarcinogenesis: telomerase (hTERT) reactivation [62], loss of cell cycle checkpoints by inactivation of p53/p21 pathway [63], loss of heterozygosity in insulin-like growth factor 2 receptor (IGF2R) gene [64], disruption of Rb/p16 pathway [65], increased resistance to apoptosis [66], activation of cellular growth/proliferation/development-related pathways such as MAPK pathway, ErbB receptor pathway, beta-catenin/Wnt pathway, and PI3K/Akt pathway [67,68]. However, it is still controversial whether these are early or late events in HCC initiation and progression.

microRNA (miRNA) profiling studies have identified dysregulated miRNAs in HCC tumors, and some of them are assumed to be involved in the process of hepatocarcinogenesis [69]. miR-122, down-regulated due to DNA methylation-mediated silencing, is assumed to be involved in dedifferentiation and tumorigenesis by activating CCNG1 [70]. Silencing of miR-122 was associated with increased tumor invasiveness, elevated alpha-fetoprotein expression, and higher HCC grade [71]. Restoration of miR-122 expression suppressed tumorigenicity of HCC and aflatoxin-transformed cells [72]. Therapeutic delivery of miR-26a suppressed tumorigenesis in a murine liver cancer model [73].

Preneoplastic lesions in cirrhotic liver

Relative difficulty in imaging-based follow-up and sampling of preneoplastic lesions in the liver has made molecular assessment of the sequential carcinogenic process more challenging. In addition, there has been diversity and inconsistency in histological diagnostic criteria and terminology (such as adenomatous and atypical adenomatous hyperplasia) for preneoplastic lesions and early HCC between pathologists, especially between East and West [74,75]. Recently emerging international guideline classifies clinically detectable preneoplastic lesions into low-grade dysplastic nodule (L-DN or LGDN) or high-grade dysplastic nodule (H-DN or HGDN) [76], which provides the basis of molecular interrogation [77,78]. L-DN, occasionally accompanied with large cell change, shows more benign clinical behavior, whereas H-DN, as a potential HCC precursor, could exhibit cytologic atypia called small cell change and nodule-in-nodule appearance, in which the subnodule represents dedifferentiation of parent nodule (i.e., well-differentiated/early HCC). Histological features of early HCC include “stromal invasion”, invading tumor cells into intra-nodule portal tract. It may be helpful to clarify corresponding neoplastic lesions in animal models of hepatocarcinogenesis to better understand the mechanism of sequential malignant transformation of hepatocytes.

APPROACHES TO HCC PREVENTION

Cancer prevention is grouped into the following categories: (1) “primary prevention” to prevent exposure to risk factors, (2) “secondary prevention” to prevent cancer development in patients with risk factors, and (3) “tertiary prevention” to prevent cancer recurrence in patients curatively treated for initial cancer but not for the risk factor [79-82] (Table 1). In the field of hepatology, “primary prevention” and “secondary prevention” sometimes also refer to prevention of initial and recurrent HCC, respectively [16,83].

Primary prevention has been proven as an effective measure, whereas there are still no established secondary and tertiary prevention therapies. Interferon has been extensively tested in viral hepatitis-related HCC as proof-of-principle therapy, although there is still need for more potent and less toxic treatment.

Primary prevention

Primary prevention aims to eliminate or reduce exposure to etiological agents, and could be immediately effective especially in developing countries [84].

Viral hepatitis-related HCC—One prominent success is the population-based universal infant vaccination for HBV, which has shown to be effective in preventing neonatal HBV infection from infected mother (vertical transmission). A cross-sectional study enrolling 1515 healthy Taiwanese children revealed that the overall prevalence of HBV surface antigen seropositivity decreased from 9.8% to 1.3% over a decade after introducing the vaccination program [85]. Furthermore, annual HCC incidence in children 6 to 9 years old declined from 0.52 to 0.13 [86]. HCV vaccination is as of yet unavailable due to the high variability in the viral genomic structure, the large number of quasispecies, and the lack of a neutralizing antibody [35]. Therefore, current efforts focus on preventing new infections by educating on safe injection practice, screening donated blood products, and systematically identifying mostly asymptomatic HBV- or HCV-infected individuals with screening.

Non-viral hepatitis-related HCC—Elimination of the food contamination may be an effective public health strategy to reduce HCC [87]. Post-harvest intervention was effective to reduce AFB_1 intake in west Africa [88]. However, such intervention, which requires water and sanitation infrastructure, may be practically infeasible in many resource-poor countries [34,82,87]. Strategies to reduce other risk factors such as alcohol abuse, obesity, and diabetes are also needed especially in developed countries. Dietary iron overload as a result of drinking habit may also be preventable [35].

Secondary prevention

Secondary prevention aims to prevent HCC in individuals chronically affected with the etiological agents. This approach is grouped into two categories: (1) eradication of the etiological agents (curative treatment) and (2) blockade of carcinogenesis progression in the presence of etiological agents (non-curative treatment). Complete eradication of HBV and HCV is still challenging once chronic infection is established, and there are already more than a half billion people with chronic infection, rationalizing the non-curative approach as a practical option.

Secondary prevention should be “less toxic” to be well tolerated for long-term treatment to asymptomatic patients, and “inexpensive” considering long duration of the treatment and large size of target patient population mainly residing in poor countries. Regular tumor surveillance in patients at high-risk of HCC is a vital component of secondary prevention [49], and has been shown to improve patient survival [89,90]. However, implementation of the program in the US is still unsatisfactory [91]. Secondary and tertiary prevention will play more roles in developed countries, where exposure to etiological agents is well controlled by means of primary prevention.

HBV-related HCC—Alpha interferon and nucleoside and nucleotide analogs, suppressing HBV replication, are clinically available as antiviral therapies [92]. A series of studies collectively suggested that interferon therapy lessens the risk of HCC development [93-97]. Prolonged suppression of HBV replication by a nucleoside analog, lamivudine, reduced the risk of HBV-related HCC: the incidence of HCC was reduced 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 [98-100].

HCV-related HCC—Meta-analyses of retrospective and relatively small prospective clinical trials of interferon-based treatment have shown that sustained viral response (SVR) is consistently associated with lower risk of HCC [80,101-105] and improved patient survival [106], although SVR is achieved in only up to 50-60% of patients. Irrespective of viral clearance, it has been suggested that suppression of hepatic inflammation could delay disease progression and reduce HCC risk; biochemical response, i.e., normalization of liver

enzymes such as alanine aminotransferase (ALT), achieved by either interferon, glycyrrhizin, or ursodeoxycholic acid (UDCA), have been suggested to reduce HCC risk [80,105,107-112]. HCC risk was reduced in patients with more advanced fibrosis/cirrhosis in large randomized controlled trials of maintenance low-dose interferon (hepatitis C long-term antiviral treatment against cirrhosis [HALT-C] trial and Evaluation of PegIntron in Control of hepatitis C Cirrhosis [EPIC3] trial) [113,114]. Despite the plausible chemopreventive effect of interferon, the modest effect, poor tolerability (nearly 40% of participants could not complete full regimen in the HALT-C trial), and excess mortality [115] have drawn concern for its wide applicability for chemoprevention especially in Western countries. In some Asian countries such as Japan, antiviral therapy mainly with interferon is recommended as preventive therapy in the practice guideline [116]. *HFE* gene mutations, in particular H63D, were associated with increased SVR [117]. Of note, SVR does not preclude the necessity of HCC surveillance because the patients are still at risk of developing HCC [118].

Aflatoxin B_1-related HCC—Chlorophyllin, water-soluble salts of natural chlorophylls, acts as an interceptor molecule forming tight molecular complexes and reduces carcinogenic activity of AFB_1 *in vivo* [119], acts as an antioxidant [120], and serves as a potent inhibitor of cytochrome P450 enzymes from bioactivation of the carcinogen. A clinical trial in Qidong, China confirmed that chlorophyllin reduced urinary level of aflatoxin-N^7-guanine adducts, a biomarker of biological effectiveness of the therapy [121], warranting further assessment of its long-term effect and safety profile in a larger patient cohort. Supplementary diet with chlorophylls-rich foods such as spinach and leafy green vegetables may be an alternative. Oltipraz, a dithiolethione originally developed as an anti-schistosomal drug, potently induces enzymes detoxifying AFB_1 such as glutathione-S-transferase that enhance phase II detoxification [122-126], and inhibits phase I enzymes such as CYP1A2 and CYP3A4, that produce carcinogenic metabolite [127]. Clinical trials of oltipraz conducted in Qidong showed a 2.6-fold increase in urinary excretion of aflatoxin-mercapturic acid, a detoxification product of reactive, DNA-damaging metabolite [128]. However, a follow-up study failed to show any change in the urinary marker [129]. In addition, the high cost of its synthesis and safety issues raised concerns with its further development, and a second generation dithiolethione analog, 3H1,2-dithiole-3-thione (D3T) is now under evaluation [35,126,130].

Alcohol- and metabolic disorder-related HCC—It is not clear whether termination of alcohol abuse reduces HCC risk in alcoholic cirrhosis despite survival benefit [36,131]. There is also no clear evidence that correction of obesity and diabetes reduces HCC, suggesting the need to determine benefit of lifestyle adjustment, weight loss, bariatric surgery, and treatment of diabetes on HCC risk. Several pharmaceutical interventions have also been considered, including insulin-sensitizing agents (e.g., troglitazone, rosiglitazone, and pioglitazone) that upregulate peroxisome proliferator-activated receptor gamma (PPAR-gamma), lipid-lowering drugs (e.g., 3-hydroxy-3-methylglutaryl coenzyme A reductase, fibrates), cytoprotective agents (e.g., UDCA), and anti-oxidants (e.g., vitamin E, iron depletion, betaine, S-adenosyl-methionine, N-acetylcystein, and probucol) [132].

Iron overload-related HCC—A cohort study with long-term clinical observation showed that phlebotomy/venesection (iron depletion therapy) could be effective to reduce HCC incidence and prolong survival in GH [133]. A population-based study suggested that HCC preventive effect of phlebotomy is limited in patients with advanced fibrosis [40]. Homozygous *HFE* gene C282Y mutation, the risk allele of GH, is readily detectable with standard molecular assays, and targeted screening in combination with measurement of plasma transferrin has been proposed to identify the risk population subjected to HCC

surveillance and phlebotomy [134-136]. In patients with chronic hepatitis C, long-term phlebotomy also lowered serum ALT level and HCC [137-139].

HCC associated with other risk factors—In PBC with stage 4 cirrhosis, UDCA may lower HCC incidence [111,140,141]. Lack of biochemical response to UDCA was associated with relatively high risk of HCC [142]. AIH is largely responsive to corticosteroids and other immunosuppressive drugs, but no study has been conducted in the context of HCC prevention [143].

Tertiary prevention

HCC recurrence targeted by tertiary prevention is categorized into two types: (1) dissemination of primary tumor cells mostly observed within 1-2 years after curative treatment as "early" recurrence, and (2) *de novo* carcinogenesis arising from remnant cirrhotic liver that appears as "late" recurrence independently from the treated primary tumor [54,69,144,145]. Theoretically, the latter is divided into (2)-a promotion/growth of (pre)neoplastic clones that already exist but are not detected at the time of curative treatment, and (2)-b *de novo* initiation of (pre)neoplastic clones that are not present at the time of treatment.

Albeit diverse molecular mechanisms are involved in the process of carcinogenesis according to the etiological agents, fully developed HCC tumor generally present similar histological, biological, and clinical characteristics, and uniform treatments are applied irrespective of the etiology [49]. Accordingly, attempts of tertiary prevention have been made mostly on mixtures of various different etiologies mainly consisting of HBV and HCV. Several strategies have been evaluated as adjuvant (i.e., post-surgical) or neoadjuvant (i.e., pre-surgical) treatment in combination with surgical resection. In theory, all the secondary prevention measures may be utilized for tertiary prevention of "late" recurrences.

Cytotoxic agents—Cytotoxic drugs, including doxorubicin, epirubicin, 5-fluorouracil (5-FU), mitomycin C, cisplatin, uracil-tegafur (UFT), and 1-hexylcarbamoyl-5-fluorouracil (HCFU), have been assessed with systemic or transarterial administration for unresectable or advanced-stage HCC without showing any remarkable clinical benefit despite considerable toxicity in both adjuvant and neoadjuvant settings [146-149]. A tumor-selective prodrug of 5-FU, capecitabine, was well tolerated in patients with advanced HCC [150], and is being tested in an on-going clinical trial as adjuvant therapy after resection. Combination of gemcitabine (a cytotoxic nucleoside analog) and oxaliplatin (a platinum agent) are also under evaluation (Table 2).

Transarterial embolization—As adjuvant therapy, transarterial chemoembolization (TACE) demonstrated modest improvement in disease-free survival and/or overall survival [151-153]. However, application as neoadjuvant therapy could worsen clinical outcome because it could increase risk of liver failure, delay surgery, and compromise subsequent treatment by promoting tumor dissemination and development of collateral tumor arteries [148].

Transarterial radioembolization (TARE) with iodine-131 or rhenium-188 labeled lipiodol or yttrium-90 labeled microspheres (glass/resin beads) is an alternative to TACE with comparable effectiveness and better tolerability for unresectable HCC [154,155]. Small clinical trials of adjuvant therapy with ^{131}I-lipiodol-based TARE has shown improved disease-free and/or overall survival [156-159], warranting further clinical evaluation.

Adjuvant immunotherapy: interferon and others—Interferon has been intensively assessed as adjuvant therapy for surgical or ablative therapy in multiple randomized controlled trials [160-166]. These studies consistently showed a trend of reducing post-treatment recurrence or death with or without statistical significance. Interestingly, Mazzaferro et al. [165] reported positive effect of adjuvant interferon therapy only on “late” recurrence in HCV-infected individuals, suggesting that interferon suppressed initiation and/or promotion of *de novo* (pre)neoplastic clones rather than regrowth of disseminated primary tumor cells. A recent systematic review reported statistically significant benefit of interferon on tumor recurrence and patient survival, although a concern on adverse side effects was again raised [167]. Despite the limitation, tertiary prevention with interferon may be cost-effective in managing HCV-related HCC [168].

Immunosuppression after liver transplantation with sirolimus, also known as an mTOR inhibitor rapamycin, has been reported to reduce HCC recurrence and improve survival [169,170]. Adoptive immunotherapy, utilizing the patient's own lymphocytes activated with interleukin-2 and an antibody to CD3 *in vitro*, reduced post-surgical recurrence and recurrence-free/disease-specific survival, although further assessment is lacking [171]. Cancer vaccination with autologous HCC fragments has been tried in small phase 1/2 trials, and resulted in a decrease in tumor recurrence [172,173]. Purified thymus extracts (pTE) and synthetic thymic peptides (sTP) are thought to enhance anti-tumor and anti-infectious immunity in cancer patients despite limited clinical evidence [174]. A clinical trial of a peptide representing residues 32-36 of a nuclear protein, thymopoietin (thymopentin, TP5) is currently on-going (Table 2).

Vitamin A analog—As an adjuvant therapy, a synthetic acyclic retinoid (vitamin A analogue), polyprenoic acid (peretinoin, NIK-333) lowered incidence of second primary tumors (i.e., “late” recurrence) after surgical or ablative treatment of HCC [175] in a larger phase 2/3 trial[1].

Interestingly, peretinoin showed dose-dependent recurrence suppressive effect 2 years after the therapy was started. Similarly, the HCC suppressive effect of interferon was not apparent for the first several years in retrospective and prospective trials [113,176]. These observations may suggest that both peretinoin and interferon suppress very early step of carcinogenesis, i.e., initiation of (pre)neoplastic clones, and have no substantial effect on (pre)neoplastic clones that already pass a certain step of malignant transformation. The several years of lag time required to see the HCC suppressive effect may reflect a latent period for the initiated clones to become detectable by clinical diagnostic modalities. This may be worth noting in the design of HCC prevention trials.

POTENTIAL TARGETS OF HCC PREVENTION

The process of hepatocarcinogenesis includes initial exposure to etiological agents, establishment of chronic diseased condition, progression of liver fibrosis, “initiation” of (pre)neoplastic lesion with irreversible somatic alterations, and “promotion” of the initiated lesion into clinically recognizable HCC tumor (Fig. **1**). It is also important to note that “initiation” and “promotion” are assumed to take place under persisting chronic irritation and inflammation with chemicals, hormones, or other mediators produced in association with underlying chronic liver disease. Each of these processes could be regarded as a preventive target. Pharmaceutical intervention with natural or synthetic agents to block, retard or reverse any step of the carcinogenic process is referred to as chemoprevention [15].

[1]Okita et al., ASCO Meeting Abstract 2010, 28, 4024

Both curative (i.e., elimination of etiological agents) and non-curative (i.e., prevention of HCC in the presence of etiological agents) treatments could be considered as secondary or tertiary prevention therapy depending on potency, mode of administration, toxicity profile, and cost. Non-curative treatment could be directed at counteracting specific carcinogenic pathways such as cellular growth signaling, regulators of cell cycle or apoptosis, oxidative stress response, and inflammatory response, or preventing liver cirrhosis, the milieu promoting and supporting hepatocarcinogenesis. Several molecular pathways are involved in the processes of both carcinogenesis and fibrogenesis, and these pathways diversely function across different types of resident liver cells and infiltrating leokocytes, and/or even different populations of the same cell types, interacting with each other via autocrine and paracrine mediators.

To date, only anti-viral/inflammatory and iron depletion therapies are likely to exhibit clinically meaningful effect as non-curative HCC prevention therapies. Molecular targets such as miR-122 and platelet-derived growth factor pathway on activated hepatic stellate cells seem to be promising [177], although all of these experimental observations await clinical evaluation.

Therapies targeting hepatitis viruses

Therapy of hepatitis B—Interferon (interferon alpha-2a and peginterferon alpha-2a) and several nucleoside/nucleotide analogs (lamivudine, adefovir, entecavir, telbivudine, and tenofovir) are clinically available [178]. Frequent emergence of viral strains resistant to lamivudine is now well recognized, and the search for optimal regimen with other available drugs such as adefovir and entecavir is currently underway [92].

Therapy of hepatitis C—Growing molecular understanding of the HCV lifecycle has led to the development of direct acting antivirals (DAAs) targeting viral proteins, processes of entry to host cells, replication, virion assembly, and secretion, as opposed to modifying host immunity like interferon. HCV protease NS3/4A inhibitors such as telaprevir improved viral response in combination with the standard interferon-based regimen [179]. Inhibitors of other HCV proteins like NS5A (essential for viral replication and assembly) and NS5B (RNA polymerase) are in phase 1-2 clinical trials [180]. In addition, a miR-122 inhibitor [181] and a phytochemical, silymarin [182], among others are under clinical evaluation as anti-HCV agents. Host genes involved in the HCV lifecycle could be another class of preventive targets. An inhibitor of cyclophilin B (CyPB), a host factor interacting with NS5B, showed anti-HCV effect [183] and is currently being evaluated in a phase 2 trial. Recently discovered SNPs in IL28B gene (e.g., rs12979860) showed strong association with SVR [184].

Targets in liver fibrosis

Resolution/reversal of human liver fibrosis/cirrhosis has been clinically observed when etiological agents were successfully eliminated, suggesting that therapies for liver fibrogenesis could be used for secondary or tertiary prevention [185]. SVR improved histological fibrosis [186,187] and hepatic venous pressure gradient (HVPG) [188], a well-known prognostic factor and predictor of HCC development [189]. Weight loss in patients with NAFLD also results in improvement of liver fibrosis [190]. UDCA was shown to reduce liver fibrosis in patients with PBC [191]. Rapidly expanding knowledge of the etiology-specific molecular mechanisms in liver fibrosis has revealed many potential targets, although there is no established therapy yet [9,10,192-195].

Liver fibrosis is an excessive wound healing response to chronic liver injury that results in increased production and deposition of scar tissue, extracellular matrix (ECM). Dynamic

balancing between fibrogenesis (synthesis of and deposition of ECM) and fibrolysis (removal of ECM) determines liver fibrosis under complex interplay between various cell types in the liver, including hepatic stellate cells (HSCs, liver-specific pericytes), Kupffer cells (liver-specific macrophages), hepatocytes, cholangiocytes, sinusoidal endothelial cells, and infiltrating immune cells. Hepatic myofibroblasts (HMFs) play the central role as the key fibrogenic effector cells by producing ECM such as collagens (Fig. **2**). Transdifferentiation of quiescent lipid/vitamin A-storing HSCs is the major cellular source of HMFs [192,193], together with other less frequent sources such as periportal/perivascular fibroblasts [196], bone marrow [197], and epithelial-mesenchymal transition [198,199].

Pathways of stellate cell activation—HSC activation is initiated by liver injury, not necessarily accompanied with inflammation as seen in GH, and subsequent release of the following fibrogenic mediators. Reactive oxygen species (ROS) are generated through lipid peroxidation by HSCs, hepatocytes, Kupffer cells, and inflammatory cells with enhancement by ethanol, unsaturated fatty acids, and iron [200,201]. Hepatocyte death, including apoptosis and necrosis, serves as a stimuli activating HSCs [202], and is a potential therapeutic target [203]. Bacterial lipoploysaccharide (LPS) permeabilized from intestinal microbiota and endogenous ligands elicit fibrogenic response through Toll-like receptor 4 (TLR4) expressed on HSCs by inducing the major fibrogenic cytokine, transforming growth factor (TGF)-beta [204]. HCV infection induces TGF-beta through ROS production, p38 MAPK, C-Jun NH_2-terminal kinase (JNK), ERK, and nuclear factor kappaB (NF-kappaB) pathways [205], although toxicity concern has been raised to target TGF-beta pathway itself [194]. Platelets are important source of paracrine stimuli, including platelet-derived growth factor (PDGF), TGF-beta, and EGF. Activated, proliferating cholangiocytes (reactive cholangiocytes), showing characteristic ductular formation known as “ductular reaction”, stimulate HSC activation by secreting profibrogenic cytokines and growth factors such as TGF-beta, connective tissue growth factor (CTGF), PDGF, and hedgehog ligands [206,207]. Dynamic changes in ECM composition also contribute to initiate HSC activation and create positive feedback pathways by increasing deposition of collagen type I and III, fibronectin, and altering growth signaling through membrane receptors such as integrins [208,209].

Autocrine loop of PDGF is the most potent mitogenic signal, which induces expression of beta PDGF receptor. Transgenic mice expressing either PDGF-B or PDGF-C develop liver fibrosis [210,211]. Notably, the latter also developed hepatic steatosis and HCC, suggesting that the model is suitable for assessment of HCC preventive therapies. In fact, peretinoin was recently shown to repress fibrosis and HCC development in PDGF-C transgenic mice [212]. Monoclonal antibody targeting PDGF-B ligand (AbyD3263) reduced liver fibrosis in bile duct ligated mice [213]. Activated HSC-specific delivery of PDGF kinase inhibitor PAP19 (an imatinib derivative) with mannose-6-phosphate modified human serum albumin (M6PHSA) reduced collagen production in bile duct ligated rats [214]. Targeted delivery of interferon-gamma, an anti-fibrotic cytokine, was attempted using a cyclic peptide recognizing beta PDGF receptor to reduce fibrosis in carbon tetrachloride-treated mice [215]. CTGF also serves as a potent fibrogenic stimulus in TGF-beta-dependent and independent manners, and is potentially associated with hyperglycemia and hyperinsulinemia [193]. Vascular endothelial growth factor (VEGF) is induced by hypoxia due to impaired blood circulation accompanying progressive fibrotic change, and contributes to angiogenesis [216]. HSC migration to liver injury site can be driven by chemoattractants such as PDGF, MCP-1, and CXCR3 and inhibited by adenosine.

Activated HSCs/HMFs express alpha-smooth muscle actin (alpha-SMA) and myosin that increase contractility of sinusoid and entire liver, in which endothelin-1 (ET-1), nitric oxide (NO), angiotensinogen II, eicosanoids, somatostatin, and carbon monoxide play roles as regulators [217]. Matrix-degrading matrix metalloproteinases such as MMP-1 are

inactivated by tissue inhibitors of metalloproteinases including TIMP-1 produced by activated HSCs/HMFs [218,219], providing a rationale to target TIMP-1 as anti-fibrotic therapy. Collagen type I, fibronectin, proteoglycans, and other matrix constituents are produced by activated HSCs/HMFs with paracrine and autocrine TGF-beta as the most potent stimulus activating downstream Smad signaling cascade [220]. Chemokine ligands and receptors mediate both fibrogenic (CCR1, CCR2, and CCR5) and anti-fibrogenic (CXCL9 and CXCR3) signals [10].

NF-kappaB activation in HSCs/HMFs promotes liver fibrosis by direct fibrogenic effect, antiapoptotic effect, and secretion of inflammatory chemokines such as CCL2, CCL3, CXCL2, and CXCL5 attracting macrophages that contribute to further activation of HSCs [221-223]. It is suspected that NF-kappaB activation is a common theme linking liver injury, fibrosis, and initiation and promotion of HCC by creating tumor-friendly microenvironment [221]. Interleukin (IL)-32 expressed by hepatocytes was recently reported to be associated with hepatic inflammation and fibrosis in HCV infection [224]. A CCL5 receptor antagonist, Met-CCL5, reduced liver fibrosis in mice [225].

Adipokines including leptin, adiponectin, and resistin are implicated in liver fibrogenesis in NAFLD and hepatitis C [226,227]. Neuroendocrine signaling mediated by cannabinoid (CB)1 receptor is fibrogenic [228], and an antagonist CP-945598 is under clinical evaluation. Other neuroendocrine such as neurotrophins also promote fibrogenesis [229]. Suppression of heat shock protein (Hsp) 47, a collagen-specific chaperon, by siRNA delivered with vitamin A-coupled liposome, targeting retinol binding protein on HSCs, reduced fibrosis in rat models [230].

Pathways of liver fibrosis resolution—The molecular process of fibrolysis is accompanied with reversal of activated HSCs/HMFs to quiescent state or clearance of them through apoptosis or senescence. NK cell is considered to be important in the clearance with both mechanisms [231]. An angiotensin receptor inhibitor may reduce liver fibrosis by inhibiting expression of NADPH oxidase and collagen type I [232]. An angiotensin-converting enzyme inhibitor regressed fibrosis by reducing survival of activated HSCs by inhibiting NF-kappaB pathway [233]. PPAR-gamma negatively regulates HSC activation through epigenetic modification [234]. Recent studies have suggested involvement of microRNA such as miR-199, miR-200, miR-29, miR-150, miR-194, miR-132, miR-16, and miR-27 in HSC activation and inactivation [234-238].

Genome-wide transcriptomic and proteomic profiling results revealed difference between the genes involved in HSC activation *in vitro* and *in vivo* [222,239-243]. A gene-expression signature of culture-activated HSCs was enriched in human cirrhosis with poor clinical outcome, and conversely, a gene-expression signature of lethal human cirrhosis was induced in chemically-induced rodent model of cirrhosis and HCC [244,245]. This suggests that genomic profiling is a useful and convenient tool to evaluate clinical relevance of molecular dysregulations in experimental models of liver fibrosis and HCC.

Targets in inflammatory pathways

Several inflammatory pathways have been implicated in development of HCC [246,247]. Involvement of NF-kappaB pathway, especially in later stage (i.e., promotion) of hepatocarcinogenesis, has been pointed out in experimental models, although the strategy to antagonize the pathway is still not clear yet [221]. JNK, a MAPK, pathway is activated by stress and proinflammatory signals such as ROS, and suggested to be associated with promotion of HCC development [248]. A JNK inhibitor, SP600125, suppressed HCC development in chemically-treated (DEN) rats by shifting hepatocytic Smad3-mediated signal from oncogenesis to tumor suppression [249]. JNK activation in non-parenchymal

liver cells generated an inflammatory hepatic microenvironment that support HCC development [250], and JNK1 knockout resulted in decreased liver fibrosis [251]. JNK pathway exhibits cross talks with growth factor (e.g., EGF)-related signaling pathways [252]. Selective inhibition of cyclooxygenase-2 (COX2) prevents HCC in an experimental animal model [253]. Liver-specific expression of lymphotoxin (LT)-alpha and beta in mice caused hepatic inflammation and HCC, which was suppressed by inhibition of LT beta receptor [254].

Targets in cytoprotective enzyme-related pathways

Oxidative stress is often linked to HCC development in chronic viral hepatitis, alcoholic and non-alcoholic chronic liver injury. Phase 2 enzymes, including conjugating and antioxidative enzymes such as glutathione S-transferase (GST), UDP-glucuronosyltransferase (UGT), and NAD(P)H:quinone oxidoreductase (NQO1) exert cytoprotective effect by eliminating carcinogens and mutagens and enhancing cellular resistance to oxidative stress [126]. Many genes encoding such cytoprotective enzymes have antioxidant response element (ARE) in the regulatory regions, which is activated by a transcription factor, nuclear factor-E2-related factor 2 (Nrf2) [125]. Nrf2 is sequestered in cytoplasm by Kelch ECH-associating protein 1 (Keap1). Oxidative stress or activation of kinases such as protein kinase C (PKC), MAPK, PI3K could cause dissociation and nuclear translocation of Nrf2 followed by activation of the target genes. Several chemical agents are known to induce Nrf2 signaling and prevent chemically-induced HCC in animal models: phenolic antioxidant, e.g., butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and ethoxyquin; dithiolethiones, e.g., oltipraz and 3H-1,2-dithiole-3-thione (D3T); sulforaphane, an isothiocyanate derived from cruciferous vegetables; triterpenoids, a synthetic derivatives of oleanolic acid [126]. Deficiency of a pentose phosphate pathway (PPP) enzyme, TAL (encoded by *Taldo1* gene) induced oxidative stress, hepatic inflammation and HCC in mice, and these phenotypes were prevented by N-acetylcysteine (NAC) [255].

Depletion of (pre)neoplastic clones: retinoid (vitamin A) and radioembolization

Retinoid X receptor-alpha (RXR-alpha) is a nuclear receptor for retinoids, vitamin A and its functional analogs, and ligand-dependent transcription factor involved in regulation of epithelial cell growth, differentiation, development, and apoptosis. Upon ligand binding, RXR-alpha homodimarizes or heterodimarizes with retinoic acid receptors (RARs) or PPARs, and transactivates target genes regulating normal cell proliferation and differentiation [256]. RXR-alpha is phosphorylated by activation of Raf-MAPK signaling, sequestered from ubiquitin/proteosome-mediated degradation, and accumulates in liver cells to promote carcinogenesis. Use of acyclic retinoid is proposed to counteract this process, induce apoptosis, and eventually eliminate the (pre)neoplastic clones or inhibit “initiation” as “clonal deletion” therapy. In the clinical setting, an acyclic retinoid, peretinoin, has shown to reduce serum level of lectin-reactive alpha-fetoprotein factor 3, an assumed marker of hypothetical latent malignant clones [257]. Radioembolization and adoptive immunotherapy are assumed to eliminate clinically undetectable “initiated” tumor clones. A small molecule EGFR tyrosine kinase inhibitor, gefitinib, suppressed growth of initiated HCC tumors in vivo [258] and is currently under clinical evaluation in the setting of tertiary prevention (Table 2).

Other molecular targets

A multi-kinase antagonist, sorafenib, inhibits VEGFR-2/3, PDGF receptor, and B-Raf kinase, and is prescribed as a chemotherapeutic drug [13,14]. Recent experimental evidence has suggested that the drug may also have antifibrotic effects [259,260]. In addition, it has been shown to improve portal hypertention, a clinical manifestation of severe cirrhosis and a

predictor of HCC development [261], supposedly due to its antiangiogenic activity [262]. Sorafenib's role in HCC tertiary prevention is currently under clinical evaluation in a large-scale phase 3 trial [263]. This class of drugs may have potential to block activation of MAPK signaling in hepatice stellate cells.

Renin-angiotensin system (RAS) is suggested to be involved in hepatocarcinogenesis [264]. Inhibition of angiotensin-II (AT-II) by angiotensin-converting enzyme inhibitor (ACE-I) down regulates angiogenic factors such as VEGF, and ACE-I administration combined with branched-chain amino acids (BCAA) has been shown to attenuate insulin resistance-related hepatocaricnogenesis in a diabetic rat model [265].

PHYTOCHEMICALS AND NUTRITIONAL SUPPLEMENTS

Phytochemicals, plant-derived bioactive chemicals, and other dietary substances have been increasingly recognized as potential treatment options in HCC prevention [266,267]. Together with other food-derived agents and nutritional supplements, this class of drugs are referred to as complementary and alternative medicine (CAM). Some of them exert pleiotropic effect in preventing carcinogenesis, although mechanisms are not fully known. A few of them such as glycyrrhizin are incorporated in clinical practice in specific countries [267].

One challenge for this class of drug is to capture its assumedly mild effect as suggested by a large-scale controlled trial testing combinations of vitamins and minerals (retinol and zinc; riboflavin and niacin; ascorbic acid and molybdenum; beta-carotene, alpha-tocopherol, and selenium) that failed to demonstrate improvement of HCC-related mortality [268,269].

Glycyrrhizin

Glycyrrhizin, an extract of licorice root (*glycyrrhizae radix*), has similar chemical structure to cortisone. The aqueous preparation, Stronger Neo-Minophargen C (SNMC), has been used for over 35 years in Japan, and shown to lower serum aminotransferases, improve liver histology, suppress HCC development (i.e., secondary prevention) and recurrence after surgery (i.e., tertiary prevention) despite the lack of apparent antiviral activity [110,112,270,271]. Glycyrrhizin is considered to have cytoprotective effects through both antioxidative properties and immune modulating activities such as induction of interferon-gamma and stimulation of NK cells, and thereby suppress hepatic necroinflammation that leads to liver fibrogenesis and HCC development [272,273]. Glycyrrhizin is one of ingredients of a traditional Chinese herbal medicine, Sho-saiko-to (TJ-9), which also prevented HCC development and prolong patient survival in a clinical trial [274].

Vitamin K

Vitamin K is a lipid-soluble vitamin, and classified into naturally occurring vitamin K_1 and K_2 and chemically synthesized vitamin K_3 which demonstrates the most potent anti-proliferative activity in tumor cells *in vitro*. Vitamin K is required to synthesize coagulation factors, including prothrombin. Des-gamma-carboxy prothrombin (DCP, also known as protein induced by vitamin K absence II [PIVKA-II]) is an abnormal prothrombin induced by the absence of vitamin K_2 and is one of clinically used HCC tumor markers together with alpha-fetoprotein. DCP has been associated with aggressive behavior of HCC tumor such as more frequent post-treatment recurrence [275]. Metatetrenone (vitamin K_2) caused cell-cycle arrest, induced apoptosis, and inhibited growth and invasiveness of HCC cell lines *in vitro* and *in vivo* [276]. Clinically, metatetrenone suppressed HCC development in cirrhotic women and recurrence after curative treatment in small clinical trials without serious adverse effect [277-279]. The safety profile and relatively low cost would further warrant evaluation in larger clinical trial.

S-adenosylmethionine

S-adenosylmethionine (SAMe), which is available as a nutritional supplement for liver, joint, and mental health, is a major methyl donor ubiquitously involved in transmethylation reactions in the body [280]. SAMe is synthesized from methionine by the enzyme, methionine adenosyl transferase (MAT), mainly in the liver, and regulates hepatocyte growth and apoptosis [280]. In cirrhotic liver, activity of MAT is reduced accompanied with hypermethioninemia [281]. *Matla* gene knockout mice exhibited reduced SAMe biosynthesis, and developed steatohepatitis with oxidative liver injury and HCC [282]. SAMe inhibits mitogenic effect of hepatocyte growth factor (HGF), induced apoptosis in cancer cells (but not in normal liver cells), and prevented initiation of chemically-induced HCC in rats [283]. No effect was observed on growth of already establish tumor cells. A phase 2 clinical trial of SAMe is on-going with the primary endpoint assessing change in serum alpha-fetoprotein level (Table 2).

Carotenoids

Natural carotenoids such as beta-carotene are obtained from fruits and vegetables, and have been evaluated for cancer prevention as antioxidants, although clear benefit has not been observed yet [284]. Beta-carotene suppressed chemically-induced HCC in rats [285]. Mixture of cartenoids, including beta-carotene, alpha-carotene, lycopene, and orange-derived beta-cryptoxanthin, was tested in small clinical trials, and showed moderate effect to reduce HCC incidence [16].

Green tea and coffee

Epigallocatechin gallate (EGCG) is the most abundant green tea catechin polyphenol, and exerts potent antioxidative activity, inhibits tumor growth, and induces apoptosis *in vitro* [266]. The proapoptotic effect of EGCG was attributed to up-regulation of miR-16 in HCC cells [286]. In rodent HCC models induced with chemicals such as aflatoxin and diethylnitrosamine (DEN), green tea or EGCG reduced the size and number of tumor and placental glutathione S-transferase (GST-P)-positive preneoplastic foci [287,288]. EGCG reduced the number of spontaneous liver tumors in C3H/HeNCrj mice [289]. In a phase 2a trial of green tea polyphenols for individuals infected with HBV and exposed to aflatoxin, urine 8-hydroxydeoxyguanosine (8-OHdG), an oxidative DNA damage biomarker, were significantly reduced after 3 months of treatment [290]. Habit of drinking green tea reduced the risk of liver cancer development in alcohol drinkers [291].

Epidemiological studies have suggested modest effect of coffee drinking in reducing HCC risk [292,293]. Administeration of both coffee and caffeine, a methylxanthine, reduced chemically induced hepatocarcinogenesis in animal models [294,295]. Coffee induced UDP glucuronosyltransferases (UGT1A), an enzyme having antioxidative, cytoprotective, and genoprotective capabilities *in vitro* and *in vivo* in caffeine independent manner, and up-regulated glucuronidation by aryl hydrocarbon receptor (AhR) signaling and Nrf2 binding to antioxidant and xenobiotic response elements (ARE/XRE) in transgenic UGT1A mice [292].

Silymarin

Silymarin is a herbal flavonoid extracted from Lady's thistle's seeds, in which silibinin is the major active constituent. Silibinin showed anti-tumor effect through induction of cell cycle arrest and apoptosis in HCC cells [296]. Silymarin suppressed N-nitrosodiethylamine (NDEA)-induced hepatocarcinogenesis in rats [297]. An observational study in the participants of HALT-C trial showed that silymarin use was associated with reduced fibrosis progression, but effect of clinical outcomes such as development of cirrhosis complication

and HCC, and death was not evident during 5.5 years of median follow-up [298]. Silymarin is also evaluated as an anti-HCV agent.

Resveratrol

Resveratrol (3,4',5-trihydroxy-trans-stilbene) is a polyphenol found in peanuts, grapes, berries, and red wine, which has been studied for multiple chemotherapeutic and chemopreventive activities, including antioxidant effect by modulating expression and activity of inducible nitric oxide synthase (iNOS), antiproliferative effect accompanied with decreased HGF activity and cell cycle arrest, antiangiogenic effect through suppression of HIF-1a and VEGF expression, and antiinflammatory effect inhibiting NF-kappaB pathway [266,299]. Resveratrol also has proapoptotic effect through p38 MAPK and caspase-3 expression, which was enhanced by Caveolin-1 (CAV1) [300]. A carcinogen-activating enzyme, cytochrome P450 1A1 is inhibited by resveratrol [301]. In rodent models of HCC induced by DEN, grape extracts (assumed to be resveratrol-rich) inhibited development of preneoplastic loci positive for GST-P [302]. COX-2 activity, which is linked to overexpression of antiapoptotic Bcl-2, was suppressed by resveratrol without cardiotoxicity [303].

Miscellaneous agents

Other agents that have been evaluated in preclinical and clinical settings include genistein (phytoestrogen isoflavone), selenium (an essential mineral), curcumin (a polyfenol derived from spice turmeric), capsaicin (phenolic compound contained in hot red peppers), glucosinolate and indole-3-carbinol (I3C) (extract from cruciferous vegetables), and N-acetylcysteine (NAC) (a precursor of glutathione) [266,267,304]. Dithiolethiones are known as more potent inducers of conjugating and detoxification enzymes than phenolic antioxidants, and synthesized dithiolethiones, oltipraz and D3T, have been evaluated for the treatment of AFB_1-related HCC.

EXPERIMENTAL MODELS FOR HCC PREVENTION STUDY

With increasing recognition that the complex interplay between multiple cell types in the physiological hepatic microenvironment is critical in HCC development, it becomes more important to develop experimental models recapitulating relevant biological and/or clinical contexts. Whereas genetic engineering provides mechanistic insight in understanding pathogenic molecular aberrations, non-physiological dysregulation of engineered genes/pathways raises a concern. Traditional chemical- or diet-induced animal models may be more preferable in this regard, and the recent advent of genomic assays has enabled the interrogation of pathogenic molecular mechanisms even in these models. More sophisticated *in vitro* co-culture systems are also a promising tool.

In vitro models

In vitro models hold value for their relatively low costs and the high reproducibility of the results, and they can be easily adopted for high-throughput testing. Several *in vitro* models have been employed in the assessment of antifibrotic drugs: culture-activated rat or human HSCs [192,194] and immortalized human HSC cell lines such as LX-1/LX-2 [305] and TWNT-4 [306]. To better capture the multicellular nature in physiological microenvironment of the liver, precision-cut sliced liver tissue culture has also been utilized to study HSC biology associated with liver fibrogenesis [307-309] and to test antifibrotic drugs such as pentoxifylline, imatinib (Gleevec, PDGF receptor antagonist), and dexamethasone [310], and antioxidant/hepatoprotective activity of lichen Usnea ghattensis [311]. Sliced tissue culture model also allowed genomic assessment of molecular targeted drug [312]. Emerging micro- and nano-scale bioengineering technologies have enabled the

construction of 2-dimensional or 3-dimensional multicellular hepatic microenvironment *in vitro* that could be used in future studies [313,314].

In vivo models

It is still challenging to create animal models faithfully mimicking or recapitulating at least part of the natural history of HCC development: sequential development of chronic fibrosing hepatitis, progressive cirrhosis, and initiation and promotion of HCC. Rodent models are frequently used with the use of chemicals, diets, and genetic engineering (Table 3) [315-317]. More attention has been paid to the hepatic microenvironment which supports not only hepatocarcinogenesis, but also the maintenance and progression of established cancer nodules [246,318-320].

It is critical for a model to capture relevant biological and clinical context with regard to affected genes and molecular pathways, and dosing and schedule of the treatment ideally specific to assumed etiology [315]. Chemically-treated rats have been popular because of their higher propensity for developing fibrosis and cirrhosis. In contrast, mice have recently garnered more attention because of their small size and ease in genetic modification [194]. Some of the genetic perturbations were not sufficient to induce HCC development by themselves, but could increase susceptibility to additional carcinogenic stimuli. One potential limitation of genetic engineering models is that they may not reflect physiological dysregulation of the genes in human.

Genomics studies on human specimens have revealed that non-tumor liver tissues harbor molecular information associated with higher risk of “early” or “late” recurrence [69,145]. Propensity to “early” recurrence (dissemination of primary tumor cells) could be modulated by antitumor immunity in the liver such as a shift from Th1 to Th2 cytokines [321]. Anti-tumor inflammatory response was correlated with poorer clinical outcome [322]. Genomic and other molecular information predictive of “late” recurrence (*de novo* carcinogenesis) was also identified [245]. These data may provide potential targets for secondary and/or tertiary prevention to be evaluated in relevant animal models.

Animal models of HCC development

Chemically-induced models of HCC—A series of antioxidants and phytochemicals have been tested on rodent models treated with chemicals such as diethylnitrosamine (DEN), dimethylnitrosamine (DMN), thioacetamide (TAA), carbon tetrachloride (CCl_4), and aflatoxin to induce HCC [266]. These chemicals are usually administered in drinking water, by oral gavage, by inhaled gases or by intraperitoneal injection.

DEN is a genotoxic chemical carcinogen that causes HCC formation in both mice and rats with a big diversity in the protocol, e.g., dosage, age, sex, and strain of the animals. For example, in mice, a single bolus injection of DEN into a 15-day-old mice, when hepatocyes are still proliferating, caused HCC development in 10 months [316]. DEN has traditionally been used in the “two-step” (initiation/promotion) model of hepatocarcinogensis whereby DEN is administered at a relatively high dose (typically ~200 mg/kg) as an initiating agent and phenobarbital or 2-acetylaminoflouren (2-AAF) is given as the promoter. Limitations of the “two-step” approach include (i) HCC development is slow ranging from 12 to 18 months, and (ii) HCC tumors do not develop in a setting of cirrhosis as is typically observed in human. Repeated administration of DEN however induces cirrhosis as well as HCC [323]. Interestingly, HCCs that develop in response to DEN in mice have a global gene expression signature that is similar to a molecular subclass of human HCC with poor prognosis [324,325].

DEN-treated rodent models have been frequently used to examine potential preventive agents [16,266,267]. C3H/HeN mice fed a 0.2% curcumin-containing diet had a reduction in HCC multiplicity and incidence induced by DEN [326]. Acyclic retinoid inhibited liver tumorigenesis induced by DEN given in drinking water at 40 ppm for 2 weeks in C57BLKS/J- +Lepr(db)/+Lepr(db) obese mice [327]. The synthetic retinoid, peretinoin, inhibited carcinogenesis dose-dependently by reducing TGF-alpha expression in the surrounding liver tissue and HCC in DEN rats [328]. Peretinoin also inhibited proliferation of TGF-alpha-expressing oval-like cells and activated HSCs in 3'-methyl-4-dimethylaminoazobenzene (3'-MeDAB)-treated rats [329]. Resveratrol dose-dependently reduced total number and multiplicity of tumor nodules in "two-step" DEN rats [330]. A small molecule EGFR tyrosine kinase inhibitor, gefitinib, suppressed growth of HCC tumors initiated by DEN [258].

Genetically engineered models of HCC – transgenic models—In *TGF-alpha* transgenic CD1 mice under the control of the metallothionein promoter, around 75% of the mice developed multifocal, well-differentiated HCCs after 12 to 15 months [331]. The surrounding liver tissue in this model exhibited little fibrosis or steatosis and therefore was not very reminiscent of human disease. All CD2F1 transgenic mice that express a secretable form of EGF developed HCC by 7.1 months [332]. In these mice, combination of the *Myc* gene with either *TGF-alpha* or *EGF* dramatically increased the development of HCC, although *Myc* transgene alone developed only hepatocellular adenomas [333].

HCC tumors in double *Myc TGF-alpha* transgenic mice were characterized by high genetic instability, high proliferation rates, low levels of beta-catenin alterations, and a gene expression pattern most similar to that of poorer survival group of human HCC [324]. *Myc* also enhanced HCC development in *E2F1* transgenic mice. Single transgenic mice carrying the *E2F1* gene under the albumin promoter developed HCC at a 33% incidence after 12 months [334], but when combined with *Myc* under the metallothionein promoter, HCC developed in 100% of mice after 9 months [335]. Interestingly, *Myc E2F1* transgenic mice developed HCC that had very little genetic instability, low proliferation rates, activation of beta-catenin and a gene expression pattern most similar to those of better survival group of human HCC [324]. Therapeutic delivery of miR-26a in *Myc* transgenic mice reduced proliferation of liver cancer cells and induced tumor-specific apoptosis [73]. These models might be useful to study how to prevent "early" recurrence from each of the human HCC tumor subclasses.

miR-221 is up-regulated in 70-80% of HCCs [336] where its expression is correlated with higher tumor stage and metastasis [337]. In miR-221 transgenic B6D2F2 mice under the control of the alpha1 anti-trypsin promoter, 50% of males develop HCCs after 9 months [338]. Administration of DEN caused tumorigenesis in both transgenic and wild-type male mice after 6 months, but the transgenic mice developed more and larger tumors. Interestingly, no spontaneous tumors were observed in female transgenic mice, but administration of DEN did cause tumor development in the transgenic females, but not the wild-type controls, after 9 months. Tumor development in transgenic animals could be inhibited with anti-miR-221 oligonucleotides.

The fibroblast growth factor (FGF) pathway has recently been implicated in HCC [339] and a dual VEGFR and FGF receptor (FGFR) inhibitor, brivanib, is being tested in clinical trials of HCC. Interestingly, transgenic FVB mice overexpressing FGF19 predominantly in skeletal muscle under the control of the myosin light chain promoter develop HCC in 10 months [340]. FGF19 uniquely binds to FGFR4 so the progeny of FGF19 transgenic mice bred with FGFR4 knockout mice do not develop HCC [341]. Since FGF19 expression is

associated with poor prognosis in human HCC patients [342], this might be a useful model for future studies as well.

Genetically engineered models of HCC – knockout models—Underscoring the role of inflammation in HCC development, inhibitory kappa B kinase (IKK) subunit IKK-gamma (NEMO)-mediated NF-kB activation was shown to prevent spontaneous development of steatohepatitis and HCC [343]. Liver parenchyma-specific deletion of NEMO caused NASH and HCC. These authors have also shown that MAP3-kinase TGF-beta-activated kinase 1 (TAK1) suppressed hepatocarcinogenesis by activating NF-kappaB [344]. Interestingly, in mice with liver parenchyma-specific deletion of TAK1, NEMO promoted HCC development independent of NF-kappaB.

Hepatocyte-specific knockout of *Pten* induced perisinusoidal fibrosis and steatohepatitis characterized by macrovesicular steatosis, ballooning hepatocytes and lobular inflammatory cell infiltration after 40 weeks and HCC after 78 weeks in mice [345,346]. When the mice were fed a 5% eicosapentaenoic acid (EPA)-supplemented standard chow, steatohepatitis was ameliorated and HCC development was decreased [347]. Since PTEN loss activates AKT, AKT inhibitors could also be tested for their efficacy in inhibiting HCC development in this model.

Gender disparity of HCC development in mice treated with DEN was due to decreased IL-6 production by Kupffer cells in females, which may also explain why HCC incidence is higher in male patients [348]. Male and female *IL6* knockout mice develop HCC at roughly the same rate and estrogen treatment reduces IL-6 production by Kupffer cells in male mice treated with DEN. Knockout of Dicer1 induced impaired hepatocyte survival and hepatocarcinogenesis in cooperation with additional oncogenic stimuli in mice [349].

The Hippo-Lats-Yorkie pathway is linked to tissue overgrowth and tumorigenesis in *Drosophila*. Mst1 and Mst2, the mammalian Hippo orthologs, are constitutively active in normal mouse liver where they suppress proliferative signals from Yap1, the mammalian Yorkie ortholog. The inhibitory Ser127 phosphorylation of Yap1 was lost in *Mst1/2* knockout mice causing liver overgrowth and HCC. Mst1/2 controlled Yap1 phosphorylation through a kinase other than Lats1/2 in the mouse liver. Mst1 was more important in suppressing HCC development as HCCs developed in all of $Mst1^{-/-}Mst2^{+/-}$ mice after 15 months, whereas HCCs developed in 25% of $Mst1^{+/-}$ $Mst2^{-/-}$ mice. Loss of activated Mst1 was also observed in human HCC [350].

Liver stem/progenitor cells (historically called oval cells) are also important in the development of HCC. Liver-specific deletion of the neurofibromatosis type 2 (*Nf2* or *Merlin*) tumor suppressor gene caused massive expansion of progenitor cells throughout the liver which eventually developed into HCCs and cholangiocarcinomas. Despite the link between Nf2 and the Hippo-Lats-Yorkie pathway in *Drosophila*, Nf2 is not a regulator of Yap1 in liver progenitor cells. Proliferation of $Nf2^{-/-}$ liver progenitor cells was instead driven by increased EGFR activation. Therefore, *Nf2* knockout mice might be another model to study the preventive effects of EGFR inhibitors [351].

Inactivation of the tumor suppressor KLF6 was associated with decreased survival in HCC patients. In consistent, $KLF6^{+/-}$ mice developed significantly more tumors in response to DEN. Downregulation of KLF6 was associated with increased Mdm2 expression and decreased p53 expression in both human and mouse tumors establishing a link between the KLF6 and p53 tumor suppressors [325].

The Farnesoid X receptor (FXR) belongs to a nuclear hormone receptor superfamily and plays a vital role in liver metabolism. Its expression has been reported to be down-regulated in human HCC [352] and both male and female FXR knockout C57BL/6 mice develop HCCs after 15 months [353]. Further analysis of these animals demonstrated progressive liver disease preceding HCC development that was characterized by steatosis after 6 months, inflammatory infiltration after 9 months and fibrosis between 9 and 12 months [352]. Not surprisingly, gene expression analysis demonstrated metabolic malfunction in these animals. This model seems particularly suitable for prevention studies given the progressive nature of disease.

Diet-induced models of HCC and genetic models of NAFLD and HCC—Choline-deficient diets are known to cause steatosis, fibrosis and HCC. A methionine and choline-deficient (MCD) diet causes periportal steatosis, hepatocyte necrosis and oval cell proliferation in both mice and rats [315]. However, cirrhosis and HCC develop infrequently in rodents fed this diet so its utility for HCC prevention studies may be limited. A choline-deficient, L-amino acid-defined (CDAA) diet leads to fatty liver and HCC development more reliably and has been used in rats to study NASH [354]. Hepatocyte death and regeneration are observed after two weeks and oval cell proliferation begins after 4 weeks. Bridging fibrosis results from stellate cell activation and borderline cirrhosis is recognized by 12 weeks with frank cirrhosis developing by 30 weeks. HCCs start to develop around 30 weeks and reach 100% incidence by 52 weeks [355]. N-(4-hydroxyphenyl)retinamide (4-HPR) decreased the number and size of placental GST-P-positive foci [356] and acetylsalicylic acid (ASA, aspirin) decreases the number and size of gamma-glutamyltransferase (GGT)-positive nodules [357], although direct measurement of tumor formation is more ideal than these surrogate markers [266].

Neither 4-HPR nor ASA had any effect on steatosis in the CDAA model, indicating studies aimed at determining whether inhibiting steatohepatitis can prevent HCC development are still needed. One disadvantage though is the amount of time it takes for HCC to develop. Adult Sprague-Dawley rats fed a choline-deficient, high trans-fat diet with DEN in drinking water, however, developed steatohepatitis, cirrhosis and HCC in just 16 weeks [358]. Recent studies have revealed important role of dysregulated miRNA expression in HCC development in CDAA-fed mice: TGF-beta-mediated upregulation of miR-181b promoted hepatocarcinogenesis by targeting TIMP3 [359], and miR-155, miR-221/222, and miR-21 were up-regulated and miR-122 was down-regulated at early stages of hepatocarcinogenesis [360]. A methyl-deficient diet caused down-regulation of miR-34a, miR-127, and miR-16a, assumedly targeting *E2F3, NOTCH1, BCL6, ZFHX1B*, and *BCL2*, as early changes in the process of carcinogenesis in rats [361].

Several genetic factors predisposing to NAFLD/NASH have also been studied in rodent models, including deletion of leptin (ob/ob mice), agouti gene (KK-Ay/a mice), SREBP-1c, IKK-gamma (NEMO), and PTEN [343,362-366]. Acyl-CoA oxidase knockout mice developed steatohepatitis and HCC by 15 months [367]. HCC tumors in Acox1−/− mice had gene expression patterns that were not very similar to human HCC [324]. Both dietary and genetic obesity promoted DEN-induced HCC in mice through induction of IL6 and TNF and increased hepatic inflammation and oncogenic Stat3 activation [368]. Deletion of glycine N-methyltransferase (GNMT), involved in S-adenosylmethionine (SAM) catabolism, induced fatty liver and fibrosis in mice, which was prevented by nicotinamide (NAM) [369].

Animal models of liver fibrosis

Animal models of liver fibrosis could be valuable tools to study carcinogenic microenvironment in the liver (“field effect”). However, commonly used rodent models do

not completely mimic liver fibrosis and formation of cirrhosis as seen in human with respect to the pattern and amount of extracellular matrix deposition and alterations in the vascular and histological architecture of the liver. Therefore, it is advisable that promising therapies be tested in multiple mechanistically distinct models in order to exclude model-specific artifacts [10,194].

Models of parenchymal fibrosis—Hepatic toxins such as CCl_4, TAA, and DEN induce liver injury, hepatocyte necrosis, and regeneration that lead to panlobular parenchymal liver fibrosis [194,315]. CCl_4-treated animals were originally intended to study HCC development, but they are more frequently used today as a model of fibrosis. The severe hepatocyte necrosis observed in this model is not reminiscent of human chronic liver disease; however, the fibrosis observed closely resembles the pattern observed in human [194]. Sunitinib given daily at 40 mg/kg could decrease inflammation, HSC activation and fibrosis in Wistar rats treated with inhalation of CCl_4 [370]. Similarly, the green tea polyphenol, EGCG, decreased liver injury, HSC activation and fibrosis in mice and rats treated with CCl_4 [371,372]. These studies were not carried out long enough to assess the effect of sunitinib and EGCG on HCC development. EGCG given in drinking water inhibited spontaneous hepatocarcinogenesis in C3H/HeNCrj mice [289].

Repeated exposure to low-dose DEN elicits a chronic liver disease that progresses similar to humans. DEN administered weekly at 50 mg/kg caused inflammation after 2 weeks and periportal fibrosis with occasional bridging fibrosis after 8 weeks. Regenerative nodules started to form after 10 weeks and distinct cirrhosis was apparent after 12 weeks and progressed to HCC after 14 weeks [373]. The low-dose administration of carcinogen could also be regarded as better mimicking actual clinical setting [15].

Models of biliary fibrosis—Extrahepatic bile duct ligation (BDL) has been used to generate biliary fibrosis/cirrhosis in rodent. Mortality is high in the BDL model and therefore it is relatively difficult to assess the effect of intervention on end-stage cirrhosis complications including carcinogenesis. *Mdr2* (*Abcb4*, mouse homolog of the human *MDR3*) −/− mice accumulated bile salts in intrahepatic bile ducts and developed biliary fibrosis as a result of cholangiocyte proliferation after 4 weeks [374,375]. Fibrosis progression in this model resembled human primary sclerosing cholangitis, and tumor developed after 8-10 months. In BDL model, a multikinase inhibitor sorafenib given at 40 mg/kg daily has been shown to reduce inflammation and decrease intrahepatic fibrosis [376]. Similarly, rapamycin given at 0.5 mg/kg daily decreased ECM accumulation and activated HSCs [377]. Alpha-naphthyl-isothiocyanate (ANIT)-treated rat is another model of biliary fibrosis [378].

Genetically engineered models of liver fibrosis—Liver-targeted *TGF-beta1* transgenic mice developed fibrosis after 12 weeks, but not HCC [379]. However, when given TAA in drinking water at 300 mg/L, HCC incidence increased from 40% in wild-type controls to 100% [380]. *PDGF-C* transgenic mouse model more resembled disease progression in human: the model exhibited HSC activation after 3-5 weeks and steatosis and fibrosis by 9 months. The pericellular and perivenular fibrosis in this model was reminiscent of alcoholic liver disease and NASH in human, and preneoplastic foci developed after 6 months, and 80% of the mice had HCC by 12 months [211]. However, little hepatocyte injury in this model as demonstrated by the low levels of serum aminotransferases and absence of cirrhosis and regenerative nodule may be limitations. *PDGF-B* transgenic mice similarly developed hepatic fibrosis, but not HCC [210]. One concern on these models is the transgenes were expressed in hepatocytes, whereas physiologically they are mainly produced by activated HSCs and inflammatory cells [194].

Senescence of activated HSC is known to limit the development of liver fibrosis [231,381]. Interestingly, IL-22 transgenic C57BL/6 mice were protected from CCL4-induced liver fibrosis through increased senescence of HSC [382]. IL-22 induces senescence by binding to its receptors, IL-10R2 and IL-22R1, on HSC and activating STAT3 and up-regulating SOCS3 which lead to increased expression of p53. The ability of compounds that induce HSC senescence to prevent HCC development and not just liver fibrosis remains to be tested.

Animal models to assess tumor microenvironment

Orthotopic xenograft models may allow studying tumor microenvironment that affects biological behavior of the tumor such as growth and intra- and extra-hepatic metastasis [383]. Genomic studies have identified common molecular subclasses of primary human HCC tumors that are linked to certain molecular abnormalities and associated with clinical outcome such as survival and tumor recurrence after surgery [69,384], some of which seem to be preserved in hepatoma cell lines [385-387]. Xenograft of these cells therefore may present an opportunity to study molecular subclass-specific prevention targets. Fresh human HCC samples can also be orthotopically implanted into the livers of BALB/c nude mice [388]. After 10 days, the resultant HCC tumors were resected and the mice were treated with or without sorafenib daily at 40 mg/kg. Sorafenib suppressed postsurgical intrahepatic recurrences and prolonged postoperative survival.

It is also critical to model cellular and tissue context in the liver, in which tumors develop. An orthotopic mouse model, whereby mouse hepatoma cells Hepa129 were implanted in syngenic C3H mice, was treated with or without intraperitoneal injections of TAA and oral alcohol to induce liver fibrosis [389]. Tumors that developed in the fibrotic livers were 3.7-fold larger than those in non-fibrotic controls, suggests that underlying fibrotic disease has dramatic effects on tumor progression.

CLINICAL CHALLENGE AND FUTURE PERSPECTIVE

Despite all of these potential preventive approaches, very few of them have been moved to clinical evaluation assumedly due to several obstacles (Table 2). First, clinical trials of preventive therapies may be less attractive to the pharmaceutical industry due to their longer length and the requirement for a larger trial size because a slower occurrence of clinical endpoint is expected compared to trials of chemotherapeutic drugs. In addition, the requirement for less toxicity and cost to enable long-term administration for mostly asymptomatic individuals further raises the bar. As a potential solution in general, novel trial design, namely “exploratory investigational new drug studies” or “phase 0” clinical trials, have been proposed [390,391]. Particularly in HCC prevention, it seems to be reasonable to start the assessment of promising therapy in the setting of tertiary prevention because HCC recurrence is more frequent than initial HCC occurrence [7,95]. In fact, many experimental approaches, e.g., interferon, retinoid, radioembolization, and adoptive immune therapy, were first tested as tertiary prevention therapies. In addition, higher toxicity and cost may be more justifiable compared to the situation of secondary prevention because the participants are regarded as “cancer” patients.

Second, clinical trial endpoints are uncertain especially for antifibrosis therapies. This has extra relevance because liver fibrosis-related trial endpoints may be used as surrogate markers of HCC occurrence in HCC prevention trials. Liver biopsy has been regarded as gold standard in the evaluation of hepatic fibrosis, but the problem of sampling variability and invasiveness are considered critical drawbacks [10,392,393]. Clear and widely applicable definition of clinical endpoint, as was proposed for assessment of anti-HCC drugs, is urgently needed to establish the drug development pipeline for HCC prevention

[10,394]. Surrogate biomarker of clinical outcome will be extremely useful. For example, in the field of antifibrosis drug development, substantial efforts have been taken to develop non-invasive methods, including ultrasound-based elastography, laboratory test-based scoring methods, magnetic resonance spectroscopy, and metabolic breath test, some of which are under clinical evaluation [395,396]. A panel of 7 genetic polymorphisms (Cirrhosis Risk Score) was shown to be associated with the risk of fibrosis progression in male Caucasian patients with mild chronic hepatitis C [397,398]. However, none of them has been reliably connected with long-term clinical outcomes such as HCC development (most relevant endpoint in HCC prevention) and cirrhosis complication, hepatic decompensation, and death. Genomic profiling of archived tissue specimen has been suggested as a promising approach to overcome this issue by directly linking molecular information with long-term clinical outcomes [244,245]. Although such tissue-based approach will require invasive tissue acquisition procedure, one may argue that it is well rationalized in establishing the therapy's benefit in the setting of a clinical trial [269].

One practical issue in tertiary prevention is how to distinguish between “early” and “late” recurrences, either of which is assumed to be targeted by each specific therapeutic approach. Genetic assessment of tumor clonality will provide more reliable biological evidence for this distinction, although no such test is clinically available [245,399-401]. Clinical studies have suggested that radiological and/or histological assessment perform reasonably well to discriminate “early” recurrence (intrahepatic metastasis of treated primary tumor) form “late” recurrence (*de novo* multicentric cancer) based on the assumption that the latter is hypovascular and well-differentiated while tumor size is small [402,403]. Multidisciplinary efforts to establish standardized clinical endpoints for HCC preventive trials will also need to address this type of clinically assessable endpoint.

Third, there is no systematic way to reassess antiviral or antifibrotic drugs that did not meet their primary endpoint (i.e., complete viral clearance or resolution of fibrosis) for their potential value as non-curative preventive therapy. A point of trade-off between effectiveness and toxicity/cost of the drugs will be different between applications such as antiviral/antifibrotic and preventive therapies. Such a system may facilitate efficient allocation/relocation of drugs in the management of the entire clinical course of chronic liver diseases aiming at controlling critical outcomes such as HCC development and death.

CONCLUSION

HCC prevention involves almost all fields of clinical and basic hepatology research. Therefore, efficient and functional integration of multidisciplinary efforts will be the key in translating basic science-oriented discovery to clinical practice. To this end, it will be extremely important to establish widely applicable clinical trial endpoint(s) and develop reliable biomarkers to measure clinical outcome.

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ABBREVIATIONS

AFB_1	aflatoxin B_1
alpha-SMA	alpha smooth muscle actin
BDL	bile duct ligation

CCl_4	carbon tetrachloride
CTGF	connective tissue growth factor
DAA	direct acting antiviral
DEN	diethylnitrosamine
DMN	dimethylnitrosamine
ECM	extracellular matrix
EGF	epidermal growth factor
GH	genetic hemochromatosis
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
H-DN	high-grade dysplastic nodule
HGF	hepatocyte growth factor
HMF	hepatic myofibroblast
HSC	hepatic stellate cell
IKK	inhibitory kappa B kinase
iNOS	inducible nitric oxide synthase
JNK	C-Jun NH2-terminal kinase
L-DN	low-grade dysplastic nodule
MAPK	mitogen-activated protein kinase
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NF-kappaB	nuclear factor kappa B
PDGF	platelet-derived growth factor
PPAR-gamma	peroxisome proliferator-activated receptor gamma
RAR	retinoic acid receptor
ROS	reactive oxigen species
TAA	thioacetamide
TACE	transarterial chemoembolization
TARE	transarterial radioembolization
TGF	transforming growth factor
UDCA	ursodeoxycholic acid
VEGF	vascular endothelial growth factor

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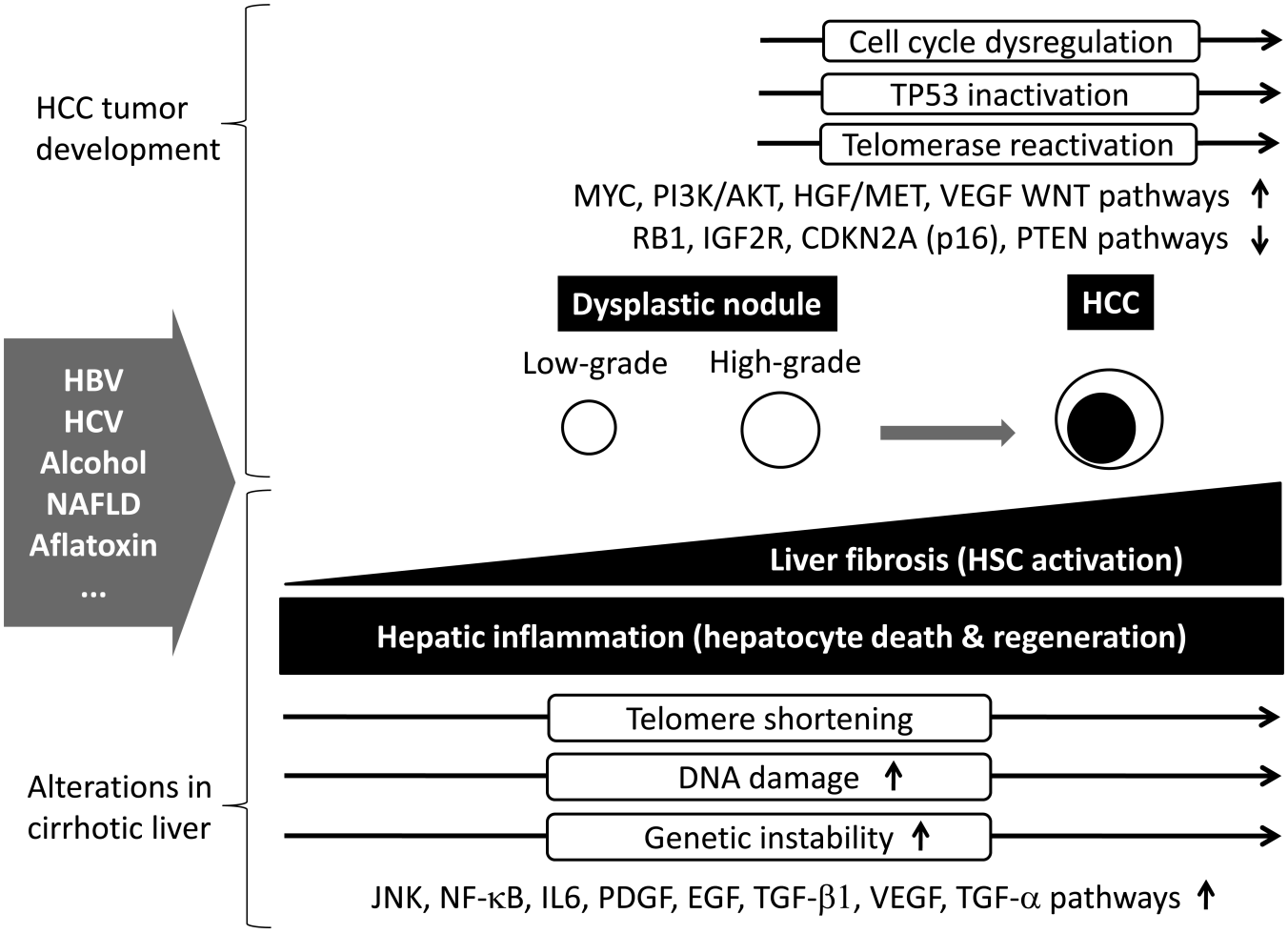

Fig. (1).

Mechanisms of hepatocarcinogenesis. Molecular pathways involved in HCC tumor development are summarized in upper panel. HCC tumor often develops as subnodule within high-grade dysplastic nodule accompanied with various molecular aberrations. Lower panel summarizes molecular alterations observed during the course of progressive liver fibrosis that leads to establishment of cirrhosis. Cirrhotic microenvironment in the liver is assumed to support initiation and promotion of hepatocarcinogenesis (so called “field effect” or “field cancerization”). HCC: hepatocellular carcinoma, HBV: hepatitis B virus, HCV: hepatitis C virus, NAFLD: non-alcoholic fatty liver disease, HSC: hepatic stellate cell

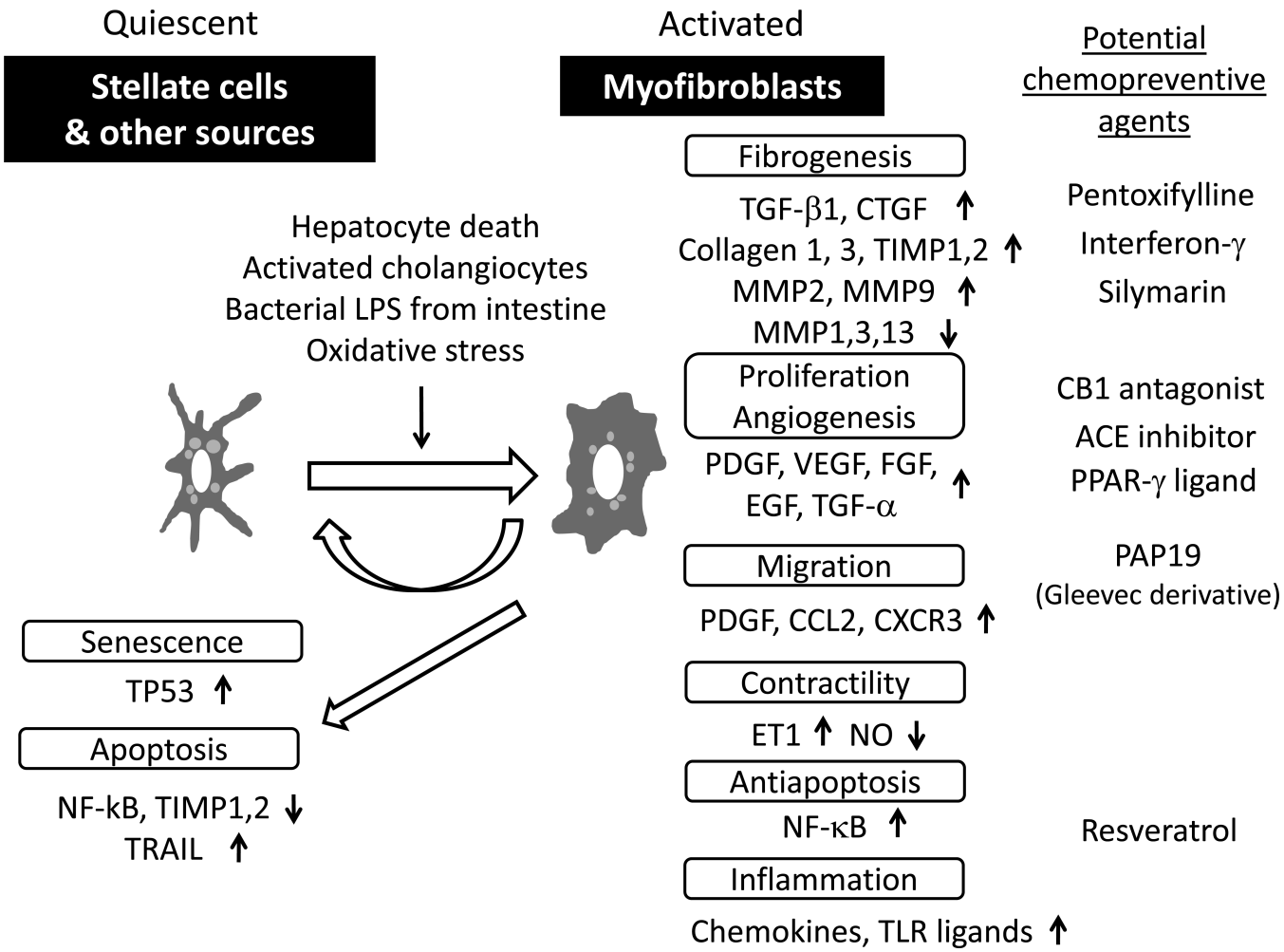

Fig. (2).

Molecular pathways in hepatic myofibroblast. Hepatic myofibroblasts, mainly derived from transdifferentiated hepatic stellate cells and periportal/perivascular fibroblasts, play major role in liver fibrogenesis. Various paracrine stimuli trigger the activation characterized by several phenotypic changes that involve specific genes and pathways (see text for details).

Table 1

Approaches to HCC Prevention

Disease etiology	Type of prevention			
	Primary	**Secondary**	**Tertiary (targeting "early" recurrence)**	**Tertiary (targeting "late" recurrence)**
Hepatitis B virus	Universal vaccination Prevention of new infection (Screening of blood products, safe injection practice)	Blood screening, HCC surveillance Antiviral therapy (interferon, nucreos(t)ide analogs) Antiinflammatory/cytoprotective therapy (glycyrrhizin, UDCA)? Antifibrosis therapy? Acyclic retinoid? Vitamin K_2?	Cytotoxic drug? Transarterial embolization? Molecular targeted drug (gefitinib)? Immune modulator (sirolimus?, thymopentin?, adoptive immunothepaty?, HCC vaccination?)	Antiviral therapy (interferon, nucreos(t)ide analogs) Antiinflammatory/cytoprotective therapy (glycyrrhizin, UDCA)? Antifibrosis therapy? Acyclic retinoid? Vitamin K_2?
Hepatitis C virus	Prevention of new infection (Screening of blood products, safe injection practice)	Blood screening, HCC surveillance Antiviral therapy (interferon, interfron + ribavirion?) Antiinflammatory/cytoprotective therapy (glycyrrhizin, UDCA)? Antifibrosis therapy? Iron depletion? Acyclic retinoid? Vitamin K_2? S-adenosylmethionine (SAMe)?	Same as above	Antiviral therapy (interferon, interferon + ribavirion?) Antiinflammatory/cytoprotective therapy (glycyrrhizin, UDCA)? Antifibrosis therapy? Iron depletion? Acyclic retinoid? Vitamin K_2? S-adenosylmethionine (SAMe)?
Dietary carcinogens (mainly aflatoxin B_1)	Reduction of food/water pollution (establishment of water and sanitation infrastructure)	Antioxidant, inducer of cytoprotective enzymes (chlorophyllin?, oltipraz?, 3H1,2-dithiole-3-thione?)	Same as above	Antioxidant, inducer of cytoprotective enzymes (chlorophyllin?, oltipraz?, 3H1,2-dithiole-3-thione?)
Alcohol	Prevention of alcohol dependence/abuse	Abstinence of alcohol intake (not effective in cirrhosis?) Correction of nutritional deficiencies	Same as above	Abstinence of alcohol intake (not effective in cirrhosis?) Correction of nutritional deficiencies
Non-alcoholic metabolic disorders (non-alcoholic fatty liver disease, obesity, type 2 diabetes)	Education on diet and physical activity Policy for healthy diet	Life style/diet modification Weight loss (diet, aerobic exercise) Control of diabetes Cytoprotective drug (UDCA?)	Same as above	Life style/diet modification Weight loss (diet, aerobic exercise) Control of diabetes Cytoprotective drug (UDCA?)
Genetic hemochromatosis	Genetic screening for high risk population	Iron depletion	Same as above	Iron depletion
Excess dietary iron	Education on dietary/ drinking habit	Modification of dietary/drinking habit	Same as above	Modification of dietary/drinking habit

Disease etiology	Type of prevention			
	Primary	Secondary	Tertiary (targeting “early” recurrence)	Tertiary (targeting “late” recurrence)
		Iron depletion		Iron depletion
Primary biliary cirrhosis		Cytoprotective drug (UDCA?)	Same as above	Cytoprotective drug (UDCA?)
Autoimmune hepatitis		Corticosteroids, immunosuppressive drugs	Same as above	Corticosteroids, immunosuppressive drugs

HCC: hepatocellular carcinoma, UDCA: ursodeoxycholic acid

“Early” recurrence: dissemination of primary tumor cells, usually observed within 1~2 years after curative treatment.

“Late” recurrence: de novo multicentric hepatocarcinogenesis independent from completely removed primary tumor, usually observed more than 1~2 years after curative treatment.

Table 2

On-going Clinical Trials of HCC Prevention

Agent/intervention	Type of agent/intervention	Type of prevention	Participants	Phase	Completion date	NCT ID
S-adenosylmethionine (SAMe)	Nutritional supplement	Secondary	Advanced chronic hepatitis C	2	Dec 2012	NCT005134 61
Phlebotomy	Iron depletion	Secondary	Compensated alcoholic cirrhosis	3	Jun 2017	NCT013427 05
PEG interferon alpha-2b + ribavirin	Immune modulator, anti-viral	Tertiary	HCV-related HCC after resection	4	Feb 2013	NCT003756 61
Interferon-alpha	Immune modulator	Tertiary	p48-positive HCC after resection	-	Jan 2011	NCT008389 68
Sirolimus	Immune modulator	Tertiary	HCC (exceeding Milan criteria) after liver transplantation	3	Aug 2013	NCT005541 25
Sorafenib (STORM trial)	Kinase inhibitor	Tertiary	HCC after resection	3	Oct 2014	NCT006927 70
Gefitinib	Kinase inhibitor	Tertiary	HCC after resection	2	Dec 2012	NCT002821 00
Thymopentin	Immune modulator	Tertiary	HBV-related HCC after resection	3	Feb 2012*	NCT004606 81
Sustained released 5-FU ± cisplatin (TACE)	Cytotoxic agent	Tertiary	HCC after resection	-	Dec 2010*	NCT008178 95
Epirubicin and lipiodol (TACE)	Cytotoxic agent	Tertiary	HCC after resection	2	Dec 2010*	NCT008200 53
Capecitabine (systemic)	Cytotoxic agent	Tertiary	HCC after resection	2/3	Jul 2012*	NCT005615 22
Gemcitabine + Oxaliplatin (systemic) (vs. doxorubicin + 5-FU + cisplatin)	Cytotoxic agent	Tertiary	HCC after liver transplantation	-	Dec 2010*	NCT011250 20
Gemcitabine + Oxaliplatin (sytemic) (vs. arterial lipiodol)	Cytotoxic agent	Tertiary	HCC after resection/ablation	3	Jun 2014	NCT004703 40

HCC: hepatocellular carcinoma, HCV: hepatitis C virus, HBV: hepatitis B virus, TACE: transarterial chemoembolization, NCT ID: National Clinical Trials Identifier

*Current recruitment status is unknown in the database From www.ClinicalTrials.gov accessed June 2012

Table 3

Experimental Models for HCC Prevention Research

Model	Presence of Liver Disease	HCC development	Preventive intervention	References
Chemically-induced models				
Diethylnitrosamine (DEN)	When dosed repeatedly, bridging fibrosis by 8 weeks, cirrhosis by 12 weeks	Varies based on species, dose, route, and frequency of administration	Gefitinib, SP600125, silymarin, resveratrol, curcumin, acyclic retinoid and peretinoin inhibit HCC development; EGCG and resveratrol reduce preneoplastic GST-P foci	249, 258, 288, 297, 302, 317, 323, 326, 327, 328, 330, 373
Carbon tetrachloride (CCl_4)	Panlobular fibrosis	HCC develops after prolonged treatment	Sunitinib decreases inflammation and fibrosis; EGCG decreases fibrosis	194, 316, 370, 371, 372
Diet-induced models				
Methionine and choline-deficient (MCD) diet	Periportal steatosis; cirrhosis rarely	HCC develops rarely		316
Choline-deficient, L-amino acid-defined (CDAA) diet	Steatosis by 1 week, bridging fibrosis by 12 weeks and cirrhosis by 30 weeks	100% incidence by 52 weeks	4-HPR decreases GST-P-positive foci; ASA decreases GGT-positive foci	355, 356, 357
CDAA diet combined with DEN	Steatohepatitis and cirrhosis	HCC develops by 16 weeks		358
Surgical Models				
Bile duct ligation (BDL)	Biliary fibrosis/cirrhosis	HCC develops by 1 month	Sorafenib reduces inflammation and fibrosis; rapamycin decreases extracellular matrix accumulation	376, 377
Orthotopic implantation	Fibrosis can be induced by concurrent treatment with thioacetamide (TAA) and oral alcohol	Injected HCCs can be either hepatoma cell lines or primary tumors from patients	Sorafenib suppressed intrahepatic recurrences and prolonged survival after tumor resection	383, 388, 389
Genetically engineered models				
EGF transgenic		100% incidence by 7 months; enhanced by Myc		332
TGF-alpha transgenic	Little fibrosis or steatosis	75% incidence by 12-15 months; enhanced by Myc		324, 331, 333
E2F 1 transgenic		33% incidence by 12 months; enhanced by Myc		324, 334, 335
TGF-beta 1 transgenic	Fibrosis by 12 weeks	No spontaneous HCC; increases TAA-induced HCC development		379, 380
PDGF-C transgenic	Steatosis and fibrosis by 9 months; no cirrhosis	80% incidence by 12 months	Peretinoin	211, 212
FGF19 transgenic	Dysplastic/neoplastic nodules	HCC develops after 10 months	FGFR4 knockout	340, 341
miR-221 transgenic		HCC development accelerated by DEN	Anti-miR-221 oligo	338
NEMO knockout	NASH characterized by steatohepatitis and periportal liver fibrosis by 8 weeks, steatosis by 6 months	HCC develops by 12 months		343, 344

Model	Presence of Liver Disease	HCC development	Preventive intervention	References
TAK1 knockout	Intrahepatic cholestasis and periportal fibrosis by 6 weeks	88% incidence by 33 weeks		344
PTEN knockout	Perisinusoidal fibrosis and steatohepatitis by 40 weeks	66% incidence by 78 weeks	Eicosapentaenoic acid reduces steatohepatitsis and HCC development	345, 346, 347
IL-6 knockout		HCC incidence in response to DEN dramatically decreased		348
Acox1 knockout	Steatohepatitis	HCC develops by 15 months		324, 367
Mdr2 knockout	Biliary fibrosis by 4 weeks with disease progressing similar to primary sclerosing cholangitis	HCC develops after 8-10 months		374, 375
Mst1/2 knockout	Hepatomegaly	HCC develops after 7-15 months		350
Nf2 knockout	Expansion of liver progenitor cells by 6-8 weeks, hepatomegaly, no inflammation or fibrosis	HCC develops after 30 weeks, HCC lung metastases after 1 year		351
KLF6 knockout		Increased HCC development in response to DEN		325
FXR knockout	Hepatic inflammation and regeneration, elevated bile acids	HCC develops after 15 months		352, 353

HCC: hepatocellular carcinoma

Viral component-induced models are detailed in [315].