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Viral hepatitis and hepatocellular carcinoma: From molecular pathways to the role of clinical surveillance and antiviral treatment

Stella L *et al*. Viral hepatitis and HCC

Abstract

Hepatocellular carcinoma (HCC) is a global health challenge, with an estimated incidence of more than 1 million cases in the following years. Due to the high prevalence in low-income countries and international migration flows, hepatitis B virus (HBV) and hepatitis C virus (HCV) infections remain the main risk factors for HCC occurrence worldwide, despite the increasing frequencies of metabolic syndrome and alcoholic liver disease. Their prevalence explains the role of HBV and HCV as the leading causes of HCC occurrence in the world. In addition, hepatitis D virus coinfection can increase the oncogenic risk in patients with HBV infection. The molecular processes underlying HCC development are complex and various, either independent from liver disease aetiology or aetiology-related. The reciprocal interlinkage among non-viral and viral risk factors, the damaged cellular microenvironment, the dysregulation of the immune system and the alteration of gut-liver-axis are known to participate in liver cancer induction and progression. Oncogenic mechanisms and pathways change throughout the natural history of viral hepatitis, with the worsening of liver fibrosis, and after the interference of HBV and HCV novel therapies. The high risk of cancer incidence in chronic viral hepatitis infected patients, compared to the healthy population and to other liver disease etiologies, makes it necessary to implement a proper surveillance, both through clinical-biochemical scores and periodic ultrasound assessment. This review aims to outline viral and microenvironmental factors contributing to HCC occurrence in patients with chronic viral hepatitis, and to point out the importance of surveillance programs recommended by international guidelines to promote early diagnosis of HCC. Furthermore, the effect of the therapies for HBV and HCV infections on HCC incidence reduction is analyzed, dwelling on the recent controversy regarding HCV direct-acting antiviral effect.

Key Words: Hepatitis C virus; Hepatitis B virus; Hepatitis D virus; Hepatocellular carcinoma; Cirrhosis; Liver

Stella L, Santopaolo F, Gasbarrini A, Pompili M, Ponziani FR. Viral hepatitis and hepatocellular carcinoma: From molecular pathways to the role of clinical surveillance and antiviral treatment. *World J Gastroenterol* 2022; In press

Core Tip: Chronic hepatitis B virus (HBV), hepatitis C virus (HCV) and D (HDV) virus infection represents a global health burden, leading to liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Among these complications, HCC accounts for 3.5% of all deaths worldwide. Thus, the understanding of the role of chronic hepatitis virus infection in HCC development is necessary in order to clarify the mechanisms underlying oncogenesis, and design future treatments for this cancer. This review outlines pathophysiological and molecular pathways that contribute to carcinogenesis in HBV, HCV and HDV chronic infection, focusing on the impact of clinical surveillance and antiviral treatment on the risk of HCC development.

INTRODUCTION

Primary liver cancer is the sixth most common cancer worldwide, accounting for 800000 new cases per year, and one of the major causes of cancer-related death in men with 745000 deaths per year. It has been estimated an increased incidence after the year 2025, with more than 1 million cases per year[1]. Hepatocellular carcinoma (HCC) represents the most common histologic type among liver cancers (70%-90%)[2]. According to recent epidemiologic studies, the mortality rate from HCC is increasing in some of European and North American countries. Indeed, HCC has been recognized as the cause of death in 54%-70% of patients with compensated cirrhosis of different etiologies[3]. Nevertheless, HCC is one of the most prevalent cancers in less developed world regions.

About 60%-70% of HCCs have been linked with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, with a total incidence of 16/100000 cases globally (Table 1)[4], while the linkage with hepatitis D virus (HDV) is less clear[5].

HCC is associated with many risk factors (Table 2), with the highest odds ratios for viral ones. Cirrhosis precedes most cases of HCC, and may exert a promoting effect *via* hepatocyte regeneration and chronic inflammation[4]. There are other environmental and genetic risk factors involved in the pathogenesis of HCC, such as excessive alcohol consumption, aflatoxin intake, diabetes, obesity, and hereditary hemochromatosis[6].

This review outlines the pathophysiology and molecular pathways that contribute to carcinogenesis in HBV, HCV and HBV/HDV chronic infection, and investigates the impact of surveillance programs and antiviral treatment on the risk of HCC occurrence.

HBV INFECTION AND HCC DEVELOPMENT

Epidemiology

HBV infection is the most widespread chronic viral infection in the world. Currently 2 billion people have been infected and more than 350 million are chronic carriers of the virus[7]. Worldwide HBV surface antigen (HBsAg) seroprevalence is 3.6%, with the highest prevalence in Africa (8.83%) and in Western Pacific Asia (5.26%)[8]. In contrast, the prevalence of chronic HBV infection is low in most of European countries, and it is estimated to be around 0.5%-0.7%[9]. Underdiagnosis and undertreatment are still unsolved issues, and public awareness is largely suboptimal, with less than 25% of infected Europeans aware of HBV transmission risk at the moment of their diagnosis[9]. Thus, chronic HBV infection is the tenth leading cause of global deaths, accounting for 3.5% of all deaths worldwide (786000 deaths per year). Half of the total liver cancer mortality in 2010 has been attributed to HBV infection[10].

Molecular pathways and HCC development in chronic hepatitis B

Chronic HBV infection is the most important cause of HCC worldwide. Prospective cohort studies show a 5-100 fold increase in the risk of developing HCC among patients with untreated chronic HBV infection[2]. A systematic review estimates a yearly HCC incidence rates of 0.2% in HBV inactive carriers, of 0.6% in those with chronic infection without cirrhosis and of 3.7% in those with compensated cirrhosis[11]. Meta-analyses

show a 15-20 times greater relative risk for HCC occurrence in HBsAg positive individuals than in HBsAg negative individuals[2].

Most of HBV-infected individuals (70%-90%) develop HCC related to the onset of cirrhosis, secondary to chronic inflammation[2]. However, in up to one-third of patients HCC occurs in the absence of cirrhosis[12]. Oncogenesis in HBV infected patients is a complex phenomenon, involving most of the cell cycle regulation molecular pathways (Table 3 and Figure 1). As a noncytopathic virus, the pathogenesis of HBV tissue damage is mainly mediated by cell-mediated immune response to HBV epitopes surface proteins: Persistent HBV replication triggers immune response against the virus, leading to genetic damage and perpetrating oxidative stress. In addition to chronic inflammation, other viral mechanisms contribute to HBV carcinogenesis involving the HBs and HBx proteins, the insertional mutagenesis caused by HBV DNA integration into the host genome, which induces chromosomal instability and alters the expression of endogenous genes, the epigenetic modifications through DNA methylation, and the regulation of microRNA (miRNA) expression[13].

There are other factors increasing HCC risk among HBV carriers, that can be divided into: Viral factors (high rates of HBV replication, long term infection, coinfection with HCV/human immunodeficiency virus/HDV, high risk genotype) and host related factors, such as genetic or demographic (older ages, male sex, Asian or African origin, family history of HCC), clinical (presence of cirrhosis), and environmental (diabetes, overweight, alcohol abuse, aflatoxin exposure)[14].

VIRAL FACTORS

Role of HBV DNA level

The risk of developing HCC strictly correlates with HBV viraemia. Multiple integrations have been detected in liver tissues, and integrated HBV sequences have been observed in almost 90% of HBV-related HCCs[13]. The Reveal-HBV study shows increased HCC-related mortality in subjects with baseline HBV DNA higher than 10^6 copies/mL, compared to those with baseline DNA lower than 300 copies/mL. A

multivariate Cox regression analysis identifies an increase in HBV DNA levels as the strongest independent predictor of death from HCC, after adjusting for age, sex, smoking, alcohol consumption and HBeAg serostatus[15]. Nevertheless, patients previously defined as "inactive carriers" (HBeAg negative, HBV DNA < 10000 copies/mL and normal liver function tests) have a 5-fold higher risk of developing HCC compared to HBsAg negative controls[16].

The integration of HBV DNA into the host genome represents a major pathogenetic pathway. The plasmid-like covalently closed circular DNA (cccDNA), derived from the conversion of HBV DNA soon after the entry into the hepatocyte, is recognized in the nucleus as a damaged product, triggering DNA repair pathways, such as the activation of cell cycle checkpoints and histone degradation, which enhances DNA recombination rates[17]. The integration of HBV DNA can contribute to hepatocarcinogenesis also by altering several cancer-relevant genes[20,26]. Viral promoter-driven human transcripts have also been identified adjacent to repetitive non-coding sequences, in particular long interspersed nuclear elements or short interspersed nuclear elements[20]. Evidence for fusion proteins have been collected for retinoic acid receptor-β and human cyclin A gene, resulting in tumor-specific chimeric proteins endowed with pro-carcinogenic functions[21].

Despite this evidence, HBV integration is random and hardly ever leads to direct oncogene activation or tumor suppressor genes inactivation. However, it is widely accepted that integration contributes to hepatocytes genetic instability, leading to clonal proliferation[22]; this can occur in HBV infected patients even before liver tissue damage is clinically evident[23].

Role of HBsAg
A study shows that the presence of HBV-DNA integration increases more than 100 times the risk for HCC occurrence among HBsAg carriers compared with HbsAg negative individuals[13]. HBsAg seems to have effects on the mitochondrial function, as it reduces the lipid b-oxidative activity of enoyl-coenzyme A hydratase short chain 1.

On one hand, this interaction induces cell apoptosis by decreasing the mitochondrial membrane potential and reduces the phosphorylation of a serine/threonine protein kinase (Akt), on the other hand it leads to an increased reactive oxygen species (ROS) production and promotes UDP-glucose ceramide glycosyltransferase which contributes to the alteration of ceramide metabolism[24]. Furthermore, HBsAg can inhibit jumping translocation breakpoint, leading to increased cell motility and decreased apoptosis[13].

Role of HBeAg

The association between HBeAg and HCC has been well established by epidemiological studies, although the pathophysiological mechanisms of HbeAg-mediated oncogenesis remain unknown. A large study investigates the effect of HBV replication on HCC development risk among 11893 Taiwanese men followed up for a mean of 8.5 years: The incidence rate of HCC is 1169 per 100000 person-years among HBsAg/HBeAg positive men, 324 per 100000 person-years for HBsAg positive HBeAg negative men and 39 per 100000 person-years among HBsAg negative men[25]. HBeAg enhances cell proliferation by means of promotion G1/S transition. Data suggests that miR-106b upregulation mediated by HBeAg is involved in the pathogenesis of HBV-related HCC by downregulating retinoblastoma (RB) gene[26].

Role of HBV preS/S proteins, oxidative stress, and interaction with endoplasmic reticulum

The preS/S open reading frame encodes three envelope proteins (S or HbsAg, M, and L proteins)[27]. Various specific mutations in the preS/S gene may imbalance surface proteins' synthesis and their consequent retention within hepatocytes' endoplasmic reticulum (ER)[13,28]. This process occurs mainly in presence of HBsAg or pre-S2 mutants: The viral proteins amassed in the ER can lead to oxidative stress, stimulate cell growth and survival signaling pathways, and cause mutation through the generation of free radicals. The induced ER stress upregulates the cytoplasmic cyclin A pathway, promoting chromosome instability through centrosome over-duplication[29].

Moreover, pre-S2 mutant protein in hepatocytes can directly interact with the c-Jun activation domain-binding protein 1, inducing hyperphosphorylation of the RB tumor-suppressor and its inactivation[13,30].

Role of HBx protein

HBx is a 154 aminoacids long protein, which is essential for HBV viral life cycle. HBx is usually expressed at low levels during the first stages of infection, but with the rise of HBV DNA integration frequency during infection, relative HBx expression can increase[31]. HBx is involved in multiple and complex molecular pathways linked to mitogen and apoptotic signaling cascades, resulting in one of the most relevant oncogenic protein in HBV proteome. It does not interact directly with DNA, but rather it acts on cellular promoters, impairs DNA repair pathways, enhances DNA replication, alters epigenetic processing, and inhibits apoptosis mechanisms. HBx effects are proven to contribute to HCC progression too, by means of angiogenesis upregulation and stimulation of cell motility.

Some preclinical studies show that HBx interacts with cell cycle regulatory pathways, activating a plethora of genes involved in mitogen signaling cascades and their downstream transcription factors, including: Nuclear factor-κB (NF-κB), phosphatidylinositol 3-kinase (PI3K) and phosphatase and tensin homolog through upregulation of alpha-fetoprotein (AFP), Notch1 and Notch4, wingless-related integration site (WNT)/β-catenin signaling, activator protein 1 (AP-1), AP-2, CCAAT-enhancer-binding protein, RNA polymerase, nuclear factor of activated T cells. The activation of these pathways leads to altered expression of growth-control genes[32]. Other significant targets of HBx are p53 family genes: The viral protein directly binds to p53 and impairs its function (p53-mediated apoptosis, p53-mediated transactivation properties, cell cycle regulation)[33].

In vitro studies have provided mechanistic insights towards the understanding of the role of HBx in HBV-mediated carcinogenesis, showing an interesting effect on DNA replication. Indeed, HBx expressing cells exhibit increased DNA replication licensing

factor, chromatin licensing and DNA replication factor 1, as well as reduced expression of geminin. The impairment in these molecules homeostatic balance leads to an increase in DNA replication[34]. Furthermore, HBx impairs DNA repair enzymes, interacting with transcription factor IIH, and deregulating all other proteins involved in base excision repair pathway[32]. HBx is also involved in HBV transcription from cccDNA and in the beginning and maintaining phase of viral replication. This leads to chromatin modulation and chromosomal instability because HBx bond with cccDNA induces the recruitment of acetyltransferases P300/CREB-binding protein (CBP)-associated factor and acetylation of histone H3, inhibiting chromatin's methylation[35]. HBx seems to induce multipolar spindle formation, chromosome segregation defects and aberrant centrosome duplication, probably by sequestrating the nuclear transport receptor Chromosomal Maintenance 1 in the cytoplasm[36]. HBx binds to the HBx interacting protein, regulating the centrosome duplication, or to the UV-damaged DNA binding protein 1, that influences the stability of proteins such as the structural maintenance of chromosome proteins 5 and 6 complex, which plays a role in nuclear DNA replication and repair[37,38].

In mitochondria HBx binds heat shock proteins 60 and 70 (Hsp60 and Hsp70), and increased levels of the resulting complex have been found in HCC cells, even if the underlying carcinogenetic mechanism has not been understood yet[39]. HBx, through miR-3928v upregulation, downregulates voltage dependent anion channel 3 (VDAC3) expression in mitochondria, leading to the loss of its tumor suppression activity[40].

HBx role on apoptosis has been controversial. On one hand, HBx upregulates B-cell lymphoma 2 (Bcl-2) and myeloid cell leukemia-1, that exert an anti-apoptotic role, and downregulates Bcl-2-associated X protein, a cytosolic protein inducing apoptosis by its interaction with VDAC3 channels on mitochondrial membrane. On the other hand, HBx stimulates apoptosis by increasing the cell surface concentration of death receptor 5 and inhibits caspase-8 inhibitor A20, that have central roles in tumor necrosis factor (TNF) α-related apoptosis-inducing ligand mediated apoptosis. Moreover, HBx increases resistance to apoptosis through forkhead box protein 4, avoiding cell death primed by

oxidative stress damage[32]. In addition, HBx controls hepatic angiogenesis, upregulating vascular endothelial growth factor (VEGF), hypoxia inducible factor 1 (HIF-1) and proangiogenic growth factor angiopoietin 2 (ANG2)[41].

HBx effects on DNA methylation and acetylation

DNA methylation and acetylation are two of the most studied epigenetic modifications, and usually occur in the early stage of HCC development. The genomic hypomethylation/hypoacetylation increase chromosome instability, while localized hypermethylation/hyperacetylation decrease the expression of tumor suppressor genes[13]. DNA hypermethylation in the promoter region of specific oncosuppressor genes has been found in HBV-related HCC, mainly due to HBx effect.

HBx upregulates DNA methyltransferases 1 (DNMT1), causing an hypermethylation of rat sarcoma (RAS) association domain family member 1 (RASSF1A), involved in cell cycle maintenance, altering its expression from the very early stages of HCC development[42]. On the contrary, the downregulation of DNA methyltransferases 3A (DNMT3A) and 3L (DNMT3L) and the recruitment of histone deacetylase 1 causes the hypomethylation of intragenic CpG islands, resulting in dedifferentiation of liver cells[43]. The upregulation of methyl-CpG binding domain protein 2 and CBP triggers a molecular pathway leading to hypomethylation of promoters 3 (P3) and 4 (P4) of insulin-like growth factor 2 gene, that stimulates cell duplication pathways as well as G1/S transition[44]. Hypomethylation has been found in the promoter region of Telomerase Reverse Transcriptase (TERT), increasing the proliferative potential of the cell, and in the promoter region of cyclooxygenase-2 (COX-2), leading to a proinflammatory condition[45]. Hypermethylation has also been found in cluster of differentiation 82, a metastasis suppressor protein involved in the p53 pathway, metastasis-associated protein 1, and protocadherin 10. Deacetylation of Cadherin-1 promoter leads to E-cadherin upregulation and progression to metastatic disease[32].

A growing number of studies identify and investigate the role of specific miRNA, small non-coding RNA molecules (up to 25 nucleotides in length) that regulate gene

expression at transcriptional and post-transcriptional levels, as mediators in the hepatocarcinogenic process. Recent publications, summarized by Sartorius *et al*[46] in a meta-analysis, have found miRNAs that are involved in HBV related HCC, leading to more aggressive cancer phenotypes by means of both tumor suppressor genes inactivation and oncogenes activation.

Role of occult HBV infection

Studies with highly sensitive polymerase chain reaction assays show that HBV DNA may persist in the liver of people who have serologic recovery from acute HBV infection, as an occult HBV infection. A systematic review identifies a modest association between occult HBV infection and the risk of HCC occurrence [risk ratio (RR) = 2.83][47]. Another case-control study performed in Hong Kong shows an increased prevalence of occult HBV infection in cryptogenic HCC[48]. None of these studies is population based, and most of them have a small sample size. Thus, the association between HBV occult infection and HCC lacks convincing evidence.

Role of HBV genotypes

HCC is more common in patients with HBV genotype C, D and F than in those with genotype A[49]. The majority of retrospective and case-control studies indicate that patients with genotype C HBV have a more severe course of liver disease, and a higher risk of HCC occurrence has been reported. A community-based Taiwanese cohort study shows that genotype C is associated with an increased risk of HCC in HBV carriers [hazard ratio (HR) = 2.35, 95% confidence interval (CI): 1.68-3.30, $P < 0.001$] as well[50]. The Eradicate-B study, by analyzing HBsAg positive patients without evidence of cirrhosis and a mean follow-up of 14.7 years, points out a higher annual incidence rate of HCC in genotype C than genotype B patients[51].

HBV genotype also influences the features of HCC. In genotype B patients, solitary HCC nodules are more frequent than in genotype C (94% *vs* 86%), but are more often associated with satellite nodules (22% *vs* 12%)[52]. Indeed, several reports show that

HBV genotype type B is associated with higher risk of HCC development in youth, whereas genotype C is associated with HCC development in the elderly[53]. Genotype D has been noticed to be prevalent in HCC patients younger than 40 years of age, as compared to other genotypes (63% *vs* 44%, *P* = 0.06). Furthermore, a study carried among Alaska native HBV infected patients shows that the HCC is prevalent in genotype F than other genotypes [odds ratio (OR) = 7.73, 95%CI: 3.69-16.4, *P* < 0.001][54]. Data about genotype E, endemic in Africa, are lacking and further studies are needed to outline the carcinogenic role of this genotype. Differently from HBV genotypes, the clinical significance of HBV subtypes is still unclear[55].

Role of HBV variants

Several mutations in the X gene of the HBV genome are frequently found in HBV mutants of individuals with HCC. Some studies show that 3'-end X gene is commonly deleted in HCC cells, leading to a C-terminal truncated HBx protein, that may have a key role in the carcinogenetic process[56]. In a Taiwanese cohort, genotype C shows a higher prevalence of BCP A1762T/G1764A variant than genotype B. Patients with BCP A1762T/G1764A variant have a higher risk of developing HCC, and a long-term follow up study reveals BCP A1762T/G1764A variant as an independent predictor for progression to HCC[72]. Mutations in enhancer II (C1653T) and elsewhere in the basal core promoter (T1753V) have also found to be associated with HCC development[57].

ENVIRONMENTAL FACTORS

Role of chronic inflammation

Chronic tissue inflammation plays a central part in oncogenesis, through various complex mechanisms. In the liver, continuous inflammation and regeneration increase the risk of hepatocarcinogenesis. Cytokines production and inflammation-mediated alteration of key signaling pathways, such as signal transducer and activator of transcription 3 (STAT3) and NF-κB pathways, play an important role as well[13,58].

The most common immune alterations found during HBV infection are: Cytokines production [such as tumor growth factor (TGF-β), interleukin (IL)-4, IL-10, IL-12, IL-13], decreased CD4+ and CD8+ cells function, Treg cells dysfunction, and innate immune response suppression[59].

CD4+ Th1 and CD8+ cells play an important role in cell cycle regulation and death control of cancer cells, thus T cells dysfunction can lead to a higher risk of HCC development[60]. The impairment of HBV-specific CD8+ T cell functions in patients with chronic hepatitis B is mainly shown by the high expression levels of inhibitory receptors such as cytotoxic T-lymphocyte antigen 4, programmed cell death protein (PD) 1 and T cell immunoglobulin and mucin-domain containing (TIM) 3. CD8+TIM-3+ T cells are functionally exhausted because of the continuous immune activation and are unable to effectively produce cytokines and exert anti-viral activity[60]. Additionally, non-specific CD8+ T cells with memory phenotypes secrete interferon (IFN) γ, recruiting hepatic macrophages, which promote HCC through the secretion of TNF-α, IL-6 and Monocyte Chemoattractant Protein 1[61]. The frequency of circulating CD4+ T follicular helper cells decreases, and their function results impaired during disease progression in patients with HBV-related HCC; CD4+ cells infiltrate seems to be significantly reduced in HCC tumor regions compared to non-tumor regions[62].

Natural killer (NK) cells from HCC patients show significant increase in the expression of miR-146a, which downregulates NK cell's function and leads to a decreased IFN-γ and TNF-α production. Activation of non-virus-specific cells may result in widespread inflammation, promoting HCC development. NK cells promote hepatic stellate cells (HSCs) activation in liver fibrogenesis through the production of the inflammatory cytokines IL-4 and IL-13[63]. HSCs enhance the recruitment of regulatory T cells (Tregs) in the liver, increasing the fibrotic processes and therefore hepatocarcinogenesis. Indeed, Tregs show enhanced suppressive function by PD-1 over-expression, leading to a more immunosuppressive and exhausted cellular microenvironment in HBV-related HCC compared to non-virus-related HCC[64]. Thus,

HBV-induced immune imbalance is a major risk factor in the pathway of HCC development.

Role of the gut microbiota

The link between the gut-liver axis and HCC has been studied in animal models and small cohorts of patients, based on the theories that inflammation related to chronic liver disease causes dysbiosis and intestinal barrier dysfunction. Translocation of bacterial products in portal and systemic circulation can trigger molecular pathways leading to inflammation, in particular lipopolysaccharide (LPS) from Gram-negative bacteria wall activates the Toll-like receptor (TLR) 4 causing an increase in inflammatory cytokines production. In turn, cytokines are linked to the activation of signaling pathways of cell proliferation, such as Janus kinase (JAK)/STAT3[65]. A well characterized pattern of inflammatory cytokines and chemokines has been associated with cirrhosis and HCC, this last characterized by increased plasmatic levels of IL-8, IL-13, C-C motif chemokine ligand (CCL) 3, CCL4 and CCL5[66]. This correlates alteration in gut microbiota with replication and immortalization of HCC cells. Indeed, several studies suggest that the gut microbiota might play a key role in the process. The inflammatory role of LPS is in fact mediated by TLR4: TLR4 knock-out mice show a much lower incidence of HCC[67]. Some other studies demonstrate that diethylnitrosamine (DEN)-exposed mice have an increased abundance of Gram-negative bacteria, and, therefore, of LPS (*Escherichia coli*, *Atopobium*, *Collinsella*, *Eggerthella*, and *Coriobacterium*)[68]. High-dose probiotics administration in these animals is able not only to reverse dysbiosis, but can reduce the number and size of cancer nodules as well.

There are limited studies comparing the gut microbiota of HBV-related HCC and non-HBV non-HCV related HCC. A small Asian study enrolls 90 patients, divided in three groups: HBV related HCC (B-HCC), non-HBV non-HCV related HCC (NBNC-HCC), and healthy controls. The gut microbiota of B-HCC patients is more heterogeneous, with higher bacterial alpha diversity indices, whereas those of healthy

controls and NBNC-HCC patients exhibits similar features. However, butyrate-producing bacteria (genus *Ruminococcus*, *Feacalibacterium*, *Clostridium*) present heterogeneity in B-HCC and NBNC-HCC in the study[69]. In addition, a set of *Bifidobacterium* species is found to mark predominant dysbiosis in HBV cirrhosis patients[70]. More studies are needed to clarify the possible oncogenic role of the gut microbiota related to HBV infection in the pathogenesis of HCC.

HCC surveillance in patients with chronic hepatitis B

Eastern and western hepatological societies have the difficult task of identifying patient populations at high risk for HCC occurrence, in whom clinical and radiological surveillance is mandatory (risk above threshold, usually cumulative incidence > 1.5% yearly) and other populations where there is no certain benefit in carrying out the surveillance programs (risk unknown or below threshold)[71]. Based on these considerations, surveillance must include patients affected by cirrhosis of all causes and a subset of non-cirrhotic HBV carriers (Table 3). HCC screening and surveillance is recommended by the American Association for the Study of Liver Diseases (AASLD) guidelines in patients with cirrhosis of any cause and non-cirrhotic HBV carriers at higher risk (*i.e.,* Asian men older than 40 years old, Asian women older than 50 years old, patients with a family history of HCC, and African or North American Blacks of any age)[72,73]. The European Association for the Study of the Liver (EASL) guidelines also include non-cirrhotic patients with high or medium risk calculated with the PAGE-B score[74,75]. The Japan Society of Hepatology (JSH) differentiates "extremely-high-risk patients" (hepatitis B cirrhosis) in whom surveillance is recommended every 3-4 mo and "high-risk patients", in whom surveillance is recommended every 6 mo, and "risk factors other than hepatitis virus infection or liver cirrhosis" in whom the benefits of surveillance is uncertain[76,77].

Risk scores

As individual HCC risk factors cannot adequately classify patients with chronic hepatitis B according to their HCC risk, several risk scores have been developed for those undergoing viral treatment with nucleotide analogues: CU-HCC, CAG-HCC, REACH-B, PAGE-B, modified PAGE-B (mPAGE-B)[78]. CU-HCC, GAG-HCC and REACH-B have been validated in a cohort of Asian patients with chronic hepatitis B, 22% of whom with cirrhosis, treated with entecavir (ETV): The 5 year HCC cumulative incidence rates were 12.9% in cirrhotic patients and 2.1% in non-cirrhotic ones, while the accuracy of CU-HCC, GAG-HCC and REACH-B scores for HCC prediction at baseline was 0.8, 0.76 and 0.71 respectively[79]. Other studies confirmed an analogue predicting value of the REACH-B score in patients treated with tenofovir[80-82].

However, CU-HCC, GAG-HCC and REACH-B predictability is proven to be poor in Caucasian subjects with chronic hepatitis B. A large study including patients treated with ETV or tenofovir showed an accuracy of 0.66, 0.74 and 0.54, respectively[83]. Subsequently, Papatheodoridis et al[84] validated the PAGE-B score on 1815 Caucasians treated with ETV or tenofovir for at least 12 mo. The PAGE-B score had the advantage of including simple variables (platelets, age and gender) and was able to stratify the risk of HCC development within 5 years in: Low (0%), for PAGE-B score < 10; medium (4%), for PAGE-B score 10-17; or high (about 16%), for PAGE-B score > 17. Kim et al[85] developed the mPAGE-B score, adding serum albumin to the PAGE-B parameters, and validating the score on Asian populations. The mPAGE-B differentiated the 5-years HCC risk in low (< 8) or high (> 13). Therefore, the PAGE B score seems to perform with an adequate accuracy both in Caucasians and Asians CHB patients under the current oral antiviral drugs.

Novel biomarkers
Recently, the usefulness of two novel biomarkers, HBcrAg and Wisteria floribunda agglutinin-positive Mac-2 binding protein (M2BPGi), has been evaluated for the prediction of HCC occurrence. HBcrAg is a novel biomarker of HCC occurrence and

consists of three products of the precore/core gene: HBeAg, HbcAg and p22cr, a 22kDa protein present in DNA-negative Dane-like particles. It is used in Japan to support HCC screening in patients with chronic hepatitis B. Serum HBcr levels correlate with HBV replication, serum HBV DNA, intrahepatic cccDNA levels and transcriptional activity. In a large cohort study, HBcrAg is proven to be superior to HBV DNA in predicting HCC development in treatment-naïve patients with chronic hepatitis B. Tseng *et al*[51] show that HBcrAg level is an independent risk factor of HCC in individuals with chronic hepatitis B and intermediate viral load. Another cohort study suggests a correlation between M2BPGi, a serum glycoprotein-based biomarker (glycobiomarker), fibrosis (FIB) stage and HCC. In patients with cirrhosis M2BPGi serum levels > 1.8 cut-off index (C.O.I.) are associated with higher risk of HCC development ($P < 0.001$). Although the area under the receiver operating characteristic curves for M2BPGi and AFP are similar in cirrhotic patients of all etiologies (0.77 *vs* 0.72; $P = 0.15$), M2BPGi outperforms AFP in CHB patients (0.84 *vs* 0.75; $P = 0.02$)[86]. Furthermore, M2BPGi serum level before receiving NA and 48 wk after the beginning of treatment is predictive of HCC occurrence.

The impact of therapies for chronic hepatitis B infection on HCC occurrence
Nowadays, only a few classes of drugs have been licensed and therefore approved for hepatitis B treatment: Nucleos(t)ide reverse transcriptase inhibitors (NRTIs) and IFN-α.

Lamivudine
Lamivudine (LAM) has been the first nucleoside analogue used for the treatment of chronic HBV infection. In an Asian study, the cumulative incidence of HCC is 3.9% in LAM arm *vs* 7.4% in the placebo arm ($P = 0.047$), yet the clinical benefits on disease progression and HCC occurrence are lost when patients developed resistance (11% *vs* 5% placebo)[87]. A meta-analysis of 5 studies including 1267 patients treated with LAM compared with 1022 untreated ones demonstrates that HCC incidence is reduced by

78% (2.5% *vs* 11.7%, RR = 0.22, *P* < 0.001)[87,88]. Therefore, LAM treatment seems to reduce, but not to eliminate the risk of HCC.

ETV and tenofovir

Second-generation NRTIs, ETV and tenofovir (TDF or TAF), have a potent suppressive effect on DNA synthesis mechanism during HBV replication, although they have poor effect on the intracellular concentration and activity of cccDNA, which persist for years in the infected liver, due to the long half-life and the resistant molecular structure. In an Asian study, ETV treated patients have a 63% reduction in the incidence of HCC at 5 years compared to those not treated (3.7% *vs* 13.7%, *P* < 0.001)[89]. A Greek study followed 321 patients with chronic hepatitis B under ETV for 30 mo, comparing them with 818 patients on LAM treatment: The cumulative HCC incidence in the ETV group is 4.8% *vs* 5.6% (*P* = 0.096)[49]. In another European multicenter cohort study, after a median follow-up of 39 mo, HCC develops in 71 (4.3%) out of 1666 patients with chronic hepatitis B treated with ETV or TDF; the cumulative probability of HCC results 1.3% at the first year, 3.4% at the third year and 8.7% at the fifth year after the initiation of treatment[90]. ETV and tenofovir seem to improve liver FIB, to prevent cirrhosis complications, and to reduce, though not eliminate, the risk of HCC.

IFNα

Patients with a clinical response to conventional IFNα therapy schemes show a lower incidence of cirrhosis decompensation and a decrease in HCC occurrence, with a better overall survival compared with non-responders. A recent meta-analysis of 14 trials suggests that pegylated-IFN facilitates HBsAg clearance or seroconversion in chronic hepatitis B patients[15,91]. In another study evaluating long-term outcomes of IFNα therapy in HBeAg seropositive patients by comparing 233 patients undergoing IFN-based treatment with 233 matched controls, the cumulative HCC incidence at the end of 15 years of follow-up is 2.7% *vs* 12.5% (*P* = 0.01)[92]. Finally, a meta-analysis on 2742

subjects pooled from 12 studies also shows that IFNα treatment reduces the risk of HCC occurrence by 34% (RR = 0.66, 95%CI: 0.48-0.89)[93].

HBV vaccines

HBV vaccination prevents children from becoming HBV carriers and reduces the incidence of HCC in vaccinated populations. In Taiwan, after a vaccination campaign, average incidence of HCC in children (6 to 14 years) fell from 0.70 per 100000 children in 1981-1986 to 0.57 in 1986-1990, and further to 0.36 in 1990-994 ($P < 0.01$). Moreover, after the universal HBV immunization program in 1990s, HCC incidence reduced from 0.293-0.117 per 100000 person-years[94]. Similar findings result from South Eastern Asian and Alaskan studies[95-97]. More than 70%-80% of HCC in all developing countries are linked to CHB infection, so widespread vaccination is the most important step to prevent more than 500000 HCC cases per year worldwide[98].

HDV INFECTION AND HCC DEVELOPMENT

Epidemiology

Recent studies estimate that between 20 and 72 million people are infected with HDV[99]. Indeed, the precise global prevalence of HDV infection has remained unestablished, due to the non-standardized screening programs in different nations and the inaccessibility to testing in most highly endemic areas[100,101]. In Western Europe, the prevalence of the infection has decreased in last twenty years as a consequence of ameliorated socioeconomic conditions and national vaccination campaigns against HBV[102,103].

Chronic hepatitis D is considered the most severe form of chronic viral hepatitis, leading to a rapid progression towards liver cirrhosis and a higher mortality rate, compared to other viral hepatitis. More than 10% of chronically HDV infected patients develop liver cirrhosis within 5 years from infection, and in 30 years more than 80% of patients suffer from cirrhosis decompensation[104].

Molecular pathways and HCC development in chronic hepatitis D infection

Recent cohort studies have found a risk as much as nine times higher in HDV-infected patients compared to HBV monoinfected ones, despite the common belief that HDV doesn't represent a certain major risk factor for HCC development[116-119]. Furthermore, persistent HDV replication has been proven to be a risk factor for liver disease progression to liver cirrhosis and HCC[107]. A study by the European Concerted Action on Viral Hepatitis demonstrates an increase of HCC occurrence by a factor of 3.2 in anti-HDV positive cirrhotic patients compared to negative ones[105]. Other recent studies show an increased risk of HCC for HDV/HBV coinfected patients than in HBV monoinfected ones (adjusted HR = 9.30)[108]. One large study estimates a risk of HCC occurrence significantly higher among patients with chronic HDV infection (RR = 3.9) than among those with HBV monoinfection[109]. On the contrary, an English retrospective study reports that despite the increasing prevalence of HDV infected patients in South London, liver cancer occurrence is only slightly increased between HBV monoinfected and HBV/HDV coinfected patients (OR = 1.34)[110].

In conclusion, recent findings identify HDV infection as a risk factor for HCC, even if a direct oncogenic mechanism is unlikely. So, it is uncertain whether HDV adds oncogenic effects beyond those carried by chronic inflammation, FIB, and cirrhosis. The genomic signature of HDV related HCC has been poorly understood, but several studies have pointed out some potential oncogenic mechanisms involving: Interference of HDAg in the cell cycle through STAT3 and cyclophilin, alteration of protein synthesis, oxidative stress as result of severe inflammation, aberrant silencing of tumor suppressor genes by DNA methyltransferases, and a possible epigenetic control on HBV transcription[5]. These mechanisms are summarized in Table 4 and Figure 1.

VIRAL FACTORS

Role of HDV replication levels

Even if HDV replicative activity has been related to a poor prognosis, it is still uncertain whether levels of HDV DNA increase the risk of HCC development. Some studies find

no correlation between viral load and HCC occurrence in HDV infected patients, while some others show an increased risk of cirrhosis complications in patient with a higher HDV viraemia[104]. Further investigations are needed to point out the role of HDV replication as a risk factor in liver oncogenesis.

Role of HDAg

HDAg expression alone does not appear to have oncogenic potential. Moreover, since high levels of antigen and viral RNA can lead to a cell cycle arrest in G1 phase, this mechanism can promote DNA damage and oncogenes mutation. L-HDAg plays a key role in HDV-related oncogenesis. It can stimulate inflammatory and cell growth pathways, by enhancing TNF-α production and NF-kB upregulation, triggering STAT3 downstream proteins in the JAK/STAT molecular signaling, and recruiting small mothers against decapentaplegic (Smad) 3 protein, involved in TGF-β activation[104]. In a similar way, L-HDAg downregulates glutathione S-transferase pi 1 (GSTP1), a tumor suppressor, and antagonizes c-Jun, by neutralizing its inhibitory effect on TGF-β cascade[111]. HBx and L-HDAg can act synergistically on NF-kB and JAK/STAT regulation, increasing each other's effect.

Oxidative stress

The severe inflammation occurring in HDV infection can by itself produce free radicals, through NF-kB and STAT3 pathways. In addition, L-HDAg-induced TGF-β1 can activate NADPH oxidase (Nox) 4, resulting in a further ROS levels increase[111]. Either L-HDAg and S-HDAg bind to endoplasmic reticulum stress response element (ESRE or SRE) and stimulate an increase in TNF receptor-associated factor 2, targeting proinflammatory genes in the generation of oxygen-derived reactive products[112]. Furthermore, HDAg can stimulate the promoters of immunoglobulin protein GRP78 and GRP94 targeting inflammatory genes and upregulating ROS production[111].

Even if the damage carried to the host genome by HDV through oxidative stress is indirect, it represents one of the most important oncogenic effects of the virus. Novel

therapies such as bulevirtide can stop this process, by inhibiting viral replication and consequently virus-related inflammation and oxidative stress.

Epigenetic regulation and DNA methylation/acetylation

Both HBV and HDV-related HCC enhance oncogenes and inhibit tumor suppressor genes by epigenetic regulation, mostly through DNMT1 and DNMT3b proteins, involved in the maintenance of methylation patterns in human genome. The molecular mechanism of DNMT1 deregulation in HDV infected patients has not been established yet, even if some studies identify STAT3 regulation over DNMTs protein as the culprit of DNA methylation impairment in HDV related HCC[111].

Moreover, S and L-HDAg can increase E2F transcription factor 1 promoter, leading to cell cycle deregulation (G2/M switch) and Nox4 activation[111]. HDV antigens impair the DNA acetylation as well, mainly by the recruitment of histone acetyltransferases and P300/CBP[104]. Histone acetyltransferases increase clusterin expression, prolonging cellular survival through its antiapoptotic effect[43].

Potential role on HBV replication

An epigenetic control of HDV over HBV transcription and regulation has been postulated. There are some evidences that long-lasting active proliferation of both viruses can lead to more severe disease, until HCC development[5,113].

ENVIRONMENTAL FACTORS

Potential role of inflammation

The histological examination of liver tissue of chronically HDV-infected patients shows an increase in HDAg specific T CD4+ lymphocytes, that rise gradually along the course of the infection. Recent findings suggest that hepatitis D is an immune-mediated disease, and chronic immune activation may promote hepatocarcinogenesis[5].

PATHWAYS NEEDING FURTHER INVESTIGATION

Potential role of small and long non-coding RNAs

There is increasing interest in the study of non-coding RNAs, including small non-coding RNA and long non-coding RNAs (lncRNAs). According to a recent study Y3 lncRNA results significantly downregulated in HDV-related HCC, suggesting a possible role in the pathogenesis of HDV-related HCC[114].

Impact of therapies for chronic hepatitis D infection on HCC occurrence

Recent long-term studies point out that PEG-IFN treatment is independently associated with a better clinical outcome and a longer survival. A retrospective study shows a reduced rate of liver decompensation in PEG-IFN-α treated patients, without any difference in terms of HCC development. In addition, complete loss of HDV RNA reached with IFN-α therapy does not seem to reduce the HCC occurrence rate[5,115]. Whether PEG-IFN treatment can reduce the risk of HCC development in HDV infected patients is unknown, and more studies are needed to clarify this point. Given the recent approval of bulevirtide, data on its effect on HCC occurrence is still lacking.

HCV INFECTION AND HCC DEVELOPMENT

Epidemiology

Global prevalence of HCV, based on detectability of anti-HCV antibodies, has been estimated to be 1.6%, which corresponds to almost 115 million individuals[116]. The prevalence of viremic individuals is estimated at 1% or 71 millions[117]. HCV seroprevalence varies across geographical regions, being highest in Central Asia, East Asia, North-Africa and Middle East, where more than 3.5% of the total population is affected[118]. Russia, Egypt, Nigeria, India, Pakistan and China account for more than 50% of the total viremic HCV infection[116,118], whereas Western countries contribute only for a small percentage (seroprevalence < 1%)[119]. The number of HCV-related deaths, due to liver cirrhosis decompensation or HCC, has been increased from almost 900000 deaths in 1990s to almost 1500000 deaths in 2010s, as the result of the high prevalence and the lack of therapies[120].

Molecular pathways and HCC development in chronic hepatitis C

HCV infection has been identified as one of the major risk factors for HCC development worldwide. Prospective studies show a 15-30-fold increased risk of HCC occurrence among chronically HCV infected patients compared with HCV negative ones. HCV genetic material doesn't integrate into the host genome; therefore, HCV needs continuous replication to maintain chronic infection. Despite this, HCV exerts indirect and direct effects on hepatocarcinogenesis. On one hand, the indirect effect is related to FIB and cirrhosis: The probability of HCC development rises with the stage of FIB, most of HCV-related HCCs occur in patients with advanced FIB or cirrhosis (annual rate up to 8%, average 1%-4%)[121]. On the other hand, viral factors such as HCV core protein, non-structural proteins, structural proteins, and HCV genotypes and subtypes have a direct effect on the risk of HCC development through the modulation of hepatocytes gene expression. The multifaceted HCV infection effects on cells molecular pathways and microenvironment are summarized in Table 5 and Figure 2.

VIRAL FACTORS

Role of HCV core protein

HCV core protein presents a broad intracellular distribution in host cells, suggesting a role in the modulation of multiple metabolic and replicative processes. Host proto-oncogenes and tumor-suppressors are direct targets of HCV core protein, which interact with tumor-suppressor proteins, such as p53, p73 and RB, downregulating their functions. Interaction between p73 and HCV core protein leads to inhibition of cell growth arrest mechanisms[122].

Moreover, increased TERT gene activity, typical of cells undergoing to malignant transformation, is observed in primary human hepatocytes transfected with HCV core protein. TERT expression seems to be one of the earliest neoplastic events in HCC, conferring an immortalized phenotype to transformed hepatocytes[123]. HCV core protein inhibits the expression of the cyclin-dependent kinase inhibitor p21/WAF, that

has a role in the regulation of the cell cycle and in the induction of apoptosis. In a similar way, HCV core protein activates the rapidly accelerated fibrosarcoma 1/mitogen-activated protein kinase (MAPK) proto-oncogene pathway and nonspecific hepatitis-induced cell proliferation[124].

Furthermore, overexpression of HCV core protein seems to be important for the regulation of epithelial mesenchymal transition (EMT), driving the activation of HSCs. The induction of EMT is mediated by the inhibition of E-cadherin expression and the induction of secreted frizzled related protein 1 *via* DNA methylation and histone modifications. This pathway leads to activation of WNT/β-catenin signaling[123]. Finally, HCV core protein can suppress NF-κB, impairing downstream immune response.

Indirect HCV core protein mediated damage is carried by oxidative stress through an impairment of lipid β-oxidation and alteration of intracellular lipid metabolism, which is associated with a reduction of mitochondrial electron transport chain's function. This process induces further ROS production and regulates apoptosis through inhibition of the Hsp60, leading to a TNFα-mediated apoptosis. Recently, HCV core protein has also been proven to impair mitophagy, contributing to mitochondrial damage; the resulting oxidative stress has been identified as a key trigger of hepatocarcinogenetic process[122]. Dysregulation in angiogenetic processes and upregulation of inflammatory pathways represent other targets of indirect damage mediated by HCV core protein.

Role of other viral proteins
HCV-encoded proteins have been directly involved in the oncogenic process through interaction with several signaling pathways, mainly in experimental animal models. The HCV E2 glycoprotein interacts with CD81, a cell surface marker of NK cells. This process leads to alteration of cytokines production and dysregulation of cytotoxic granules release, impairing the host immune system[14]. In addition, E2 protein leads to MAPK/extracellular signal-regulated kinases pathway activation, promoting cell proliferation and maintaining cell survival[125].

The NS2 protein can activate cyclin D/cyclin-dependent kinase 4 (CDK4), inducing cyclin E expression and stimulating cell cycle progression from G1 phase to S phase. NS2 protein is involved in HCC carcinogenesis through tumor suppressor inactivation of p53 and consequent transcriptional activation[126].

The NS3 protein is involved in tumor cell transformation, by loss of contact inhibition and growth anchorage, evasion of innate immunity, interaction with mitochondrial signaling protein, tumor suppressors inactivation (p53 and ataxia telangiectasia mutated kinase or ATM), and alteration of immunoregulatory pathways such as TLR3, retinoic acid-inducible gene I (RIG-I) and NF-κB pathways[127].

Similarly, the NS5A protein plays a role in cell cycle dysregulation through signaling pathways, immune escape, impairment of cell apoptosis, and ROS production. NS5A is involved in proliferative pathways, by the inactivation of p53, blockade of IFN signaling through JAK-STAT cascade, and inhibition of host protein synthesis through protein kinase R (PKR)[126]. In addition, NS5A protein, like HCV core protein, can inhibit TGF-β signaling through interaction with T-box brain transcription factor 1 or direct binding to TGFβ receptor 1, and through prevention of translocation of Smad proteins[128]. On the other hand, NS5A interacts with PI3K p85 regulatory subunit, that regulates apoptotic activity of PI3K and upregulates cell survival cascade, activating Akt and Twist family BHLH transcription factor 2 pathways favouring EMT, and interacts with RAS oncogene to enhance tumor cell invasiveness[129]. NS5A stimulates TLR4 activation and its signaling through homeobox protein NANOG and consequent Twist 1 activation, leading to hepatocytes dedifferentiation[126].

NS5A affects apoptosis trough downregulatory effect on TNFα-mediated apoptosis. It deactivates caspase-3 and inhibits proteolytic cleavage of death substrates known as polyADP-ribose polymerases, impairing PD cascade[126]. Moreover, HCV seems to enhance ROS formation and aberrant cell-cycle arrest, due to DNA damage, through dysregulation of ER-mediated calcium uptake, and induction of glycogen synthase kinase 3β phosphorylation, with consequent beta-catenin-dependent upregulation of c-Myc *via* NS5A[122].

Non-structural viral proteins, together with HCV core protein, play a key role in HCV mediated oncogenesis. Their multifaceted interaction with basic hepatocytes signaling pathways favors deep dysregulation of cells homeostasis and accumulation of DNA damage until malignant transformation.

Viral effect on angiogenesis

A higher vascular density has been reported in nodules of HCV-related HCC, in relation to HCC nodules in patients affected by other liver diseases. Studies show upregulation of VEGF expression in patients with HCV-related HCC compared to the control group. HCV core protein can increase cellular VEGF expression through HIF 1α transcription factors and AP-1. Similarly, core protein can induce overexpression and stabilization of HIF-1α and Ang2, which upregulate VEGF expression[130]. Moreover, HCV core protein binds androgen receptor (AR), impairing transcriptional activity by activating several signaling pathways such as PI3K/Akt and JAK/STAT3. Since AR targets VEGF gene in the liver tissue, HCV core protein upregulates VEGF expression through enhanced activity of the AR pathway[123].

Other mechanisms of HCV core-protein promoted angiogenesis rely on upregulation of COX-2 and metalloproteinases 2 and 9 (MMP-2 and MMP9)[126]. CCL20/C-C chemokine receptor type (CCR) 6 axis is directly involved in stimulating angiogenesis in the microenvironment of HCV-related HCC. *In vitro* experiments pointed out CCL20 as a direct promotor of angiogenesis, inducing endothelial cells invasion, sprouting and migration of CCR6-positive leukocytes. A study demonstrates that HCV-induced CCL20 protein expression and secretion in hepatoma cells can be diminished and even abolished by means of antiviral treatment, proving CCL20 expression to be dependent on HCV replication[131].

Role of epigenetic modifications

Epigenetic modifications in patients' liver tissue can derive both from infected hepatocytes and from virus-induced inflammatory or fibrotic responses in the cells'

microenvironment. HCV-induced histone modifications and fibrogenesis are interdependent during the course of liver disease: Epigenetic changes orchestrate fibrogenesis through the activation of HSCs. Specific epigenetic modifications have been identified in patients' liver tissue and humanized mouse's liver tissue, where no necrotic nor inflammatory response are present, therefore it's probable that part of the changes observed can be a direct consequence of HCV-hepatocyte interaction.

HCV upregulates methyltransferase DNMT1, that hypomethylates tumor suppressor genes, such as Cyclin Dependent Kinase Inhibitor 2A, GSTP1, RUNX family transcription factor 3, suppressor of cytokine signaling 1 and RASSF1A[126]. In a similar way, an upregulation of "suppressor of variegation, enhancer of Zeste, trithorax" and myeloid nervy deaf 1 domain-containing protein 3, also named SMYD3, leads to a specific hypermethylation of histone marks H3K4 and H4K5 in tumor suppressor genes, such as RASSF1A[126].

A recent study shows that chronic HCV infection induces changes in histone mark H3K27ac, which are associated with HCC risk and seem to persist after HCV cure[132]. H3K27ac is involved in pathways related to TNFα, inflammatory response, IL-2, and activator of transcription 5 signaling. Furthermore, lower levels of H3K27ac can alter pathways associated with metabolism and coagulative cascade, such as oxidative phosphorylation, impaired fatty acid regulation, or adipogenesis. Several alterations in these pathways (*e.g.*, epigenetic alteration in TNFα signaling, G2M-checkpoint, epithelial-mesenchymal transition, PI3K, Akt and mTOR) persist even after direct-acting antiviral agents (DAAs) therapy[132]

Recently, several studies outlined the role of miRNAs-mediated epigenetic alterations in HCV related carcinogenetic processes. One study examines the expression profiles of miRNA in more than 50 HCV-related HCCs by quantitative real-time polymerase chain reaction. Five miRNAs are examined: MiR-122, miR-100, and miR-10a seem to be upregulated, while miR-198 and miR-145 are downregulated up to 5-fold in tumors than in normal liver tissue[133]. MiR-122 is highly expressed in the liver of HCV infected individuals, due to its essential role in the stability and replication of HCV RNA[134].

Another study develops an *in vitro* model of HCV infection in primary human hepatocytes to evaluate the role of miRNAs: MiR-141, which targets the tumor-suppressor gene Deleted in Liver Cancer (DLC) 1, is upregulated in HCV genotypes 1a, 1b, and 2a infected cells. The rise in miR-141 levels in HCV-infected cells correlates with an inhibitory effect on DLC-1 protein and a subsequent increase in HCC development risk[122]

Role of HCV genotypes

HCV genotype 1b is associated with at least a doubled risk of HCC compared to other HCV genotypes[135,136]. HCV genotype 3 is considered a major risk factor for hepatic carcinogenesis. A multicenter Asian cohort study shows a 4.3-fold in the risk of HCC development in patients with chronic HCV genotype 3 infection, compared to other genotypes[137]. Another Asian study shows a 5-year occurrence rate of HCC of 34% in genotype 3 and of 17% in non-genotype 3 groups, respectively ($P = 0.002$); in a multivariate analysis, HCV genotype 3 infection is independently associated with a rise of HCC occurrence rates (HR = 3.54), even after adjustment for confounding factors[138]. A further study finds an approximately doubled risk of all-cause mortality and HCC in patients with HCV genotype 3 infection, related to individuals infected with other genotypes[139]. However, the exact role of HCV genotypes in hepatocarcinogenesis processes and in the worsening of liver disease still needs to be established.

ENVIRONMENTAL FACTORS

Role of chronic inflammation and FIB

Most of HCV-related HCCs occur in cirrhotic livers, following decades of chronic inflammation, underscoring the key role of virus-induced inflammatory response in hepatic carcinogenetic processes. Generally, the inflammatory response is beneficial to the host, but in HCV patients innate and adaptive immunity result ineffective. This cycle can be primed by HCV proteins, but host immune response tends to self-maintenance.

HCV interferes with various defense mechanisms: TLR3 and RIG-I gene downstream pathogen-recognition signaling pathways are blocked by the HCV protease NS3/4A; HCV proteins NS5A and core proteins can block IFN signaling interfering with JAK–STAT pathway; HCV proteins E2 and NS5A inhibit PKR, an antiviral effector that impairs protein synthesis of infected cells; HCV core protein stimulates the release of cytokines that can lead to a direct decrease in plasmacytoid dendritic cells levels among HCV-infected patients[140,141].

The persistence of chronic inflammatory response leads to the release of free radicals, such as ROS and nitric oxide (NO)[142]. NO contributes to viral persistence leading to antiapoptotic effects in hepatocytes, and may induce viral mutations and promote suppression of Th1 cells[143]. Also, it directly influences liver cell survival by preventing apoptosis through the activation of NF-κB pathway. ROS induce modifications in structure and function of cancer-related proteins and genes, including those fundamental for cell-cycle control, apoptosis, and DNA repair[144]. There are evidences that HCV-mediated inflammation is responsible of promotion of hepatic carcinogenesis through oxidative DNA damage[145].

In patients affected by HCC, Th1 dominance is progressively lost due to an increase of Th2 cells[146]. Some studies typify the microenvironment of HCC metastatic phenotype as composed by a Th2-like cytokine profile, consisting in higher levels of IL-4, IL-8, IL-10, and IL-5, and lower levels of Th1-like cytokines as IL-1α, IL-1β, IL-2, IL-12p35, IL-12p40, IL-15, TNF-α, and IFN-γ[147]. Moreover, the peripheral blood and the tumor tissue of HCC patients show an increased number of Tregs (with a positive direct correlation with HCV RNA levels). IL-10 seems to be highly expressed in HCC patients, and its presence correlates with disease progression[148].

In conclusion, inadequate immune response is a necessary factor for hepatic cells transformation, but not a sufficient one. It builds a favorable microenvironment in which all factors (etiology genetic, epigenetic, humoral and cellular inflammation, chronic liver injury) contribute to the development of FIB and liver cells proliferation.

These oncogenic factors promote DNA abnormalities that ultimately transform the hepatocytes in malignant cells.

Role of the gut microbiota

HCV-related cirrhosis is characterized by a disruption of the gut microbiota composition leading to an increased intestinal permeability and bacterial translocation. Compared to healthy individuals, bacterial alpha diversity seems to be lower in patients affected by chronic HCV infection, with a reduction in bacteria of the order *Clostridiales* and an expansion in *Streptococcus spp*. Gut dysbiosis begins to occur in the very early stages of the infection, with the transient increase in *Bacteroidaceae* and *Enterobacteriaceae*. A decrease in the abundance of *Bifidobacteriacae* and *Lactobacillaceae*, with an increase of *Bacteroidaceae* and *Enterobacteriaceae* has been reported in the gut microbiota of patients with HCV-related HCC[149,150]. Predicted metagenomics of microbial communities show an upregulation of urease gene, mainly encoded by *Streptococcus viridans* during chronic hepatic disease course, consistent with a significantly higher faecal pH than in healthy individuals[151]. A small study shows an increase in *Streptococcus salivarius* (*S. salivarius*) in oral and gut microbiota of patients with HCV-related HCC, suggesting a possible role in oncogenesis. It has been noticed that *S. salivarius* impairs and deregulates the innate immune response of epithelial cells, with a possible contribution in HCC progression[152].

DAAs treatment has proven to modulate the composition of the gut microbiota, contributing to a decrease in inflammatory markers and liver stiffness, but without effect on the gut barrier and its permeability. The eradication of the infection cannot resolve the intestinal dysfunction caused by liver cirrhosis, but can improve the pre-existing dysbiosis[153]. Therefore, alterations of the gut microbiota may be focused as a predisposing factor for liver disease progression, but further studies are necessary to establish its possible pathogenetic role in HCC development.

Role of host genetic factors

The genetic background of the host has a growing role in hepatic carcinogenesis. A systematic review found 16 genes associated with HCV-related HCC[154]. The polymorphisms identified in patients with HCV-related HCC involve genes encoding for: TGF-β1, TNF-α, mitochondrial aldehyde dehydrogenase enzyme 2, catalase, EGF, glutathione S-transferase mu 1 protein, glutathione S-transferase theta 1, HLA-Bw4, HLA-B1814 and HLA-DR11, HSPA1B, leptin receptor, IL-1b, a regulator of the p53 tumor suppressor named mouse double minute 2 homolog, UDP glucuronosyltransferase 1 family-polypeptide A7, manganese-dependent superoxide dismutase mitochondrial protein, IFN lambda 3 and lambda 4 cytokine, patatin-like phospholipase domain–containing protein 3 (PNPLA3), and MHC class I polypeptide-related sequence A (MICA/HCP5)[154-156]. Nowadays, the genetic risk score, designed using the combination of four high risk variants in the PNPLA3, TM6SF2, MBOAT7, and Glucokinase Regulator GCKR genes, estimates the inherited predisposition to accumulate liver fat (GRS) and is reported as an independent risk factor for *de novo* occurrence of HCC after DAAs treatment[157].

HCC surveillance in patients with chronic hepatitis C

As already discussed, HCC surveillance in HCV infected patients must include patients affected by cirrhosis of all causes and patients with chronic HCV infection with advanced liver FIB (Table 6). The AASLD and EASL practice guidelines recommend HCC screening and surveillance in patients with cirrhosis or with stage 3 FIB[73,75,158]. JSH still differentiates "super-high-risk patients" (hepatitis C cirrhosis) in whom surveillance is recommended every 3-4 mo and "high-risk patients", in whom surveillance is recommended every 6 mo[124].

The impact of chronic hepatitis C treatment on HCC occurrence

The utmost importance of viral clearance in the reduction of HCC occurrence among chronically HCV infected patients has been largely established. Since the first studies on IFN-based therapy, HCC incidence shows a significant difference between patients with

sustained virological response (SVR) and non-SVR patients (5.1% and 21.8%). A large meta-analysis including 31528 HCV infected patients shows that SVR is a key factor in reducing the incidence of HCC (1.5% in SVR patients *vs* 6.2% in non SVR patients at 3-8 years of follow up)[139,160].

PEG-IFNα

IFN-α was approved to treat HCV infection in the 1980s, but its efficacy was limited (SVR rates 54%-56%). A meta-analysis of 8 long-term studies on 2649 patients with chronic hepatitis C demonstrates that HCC occurrence is significantly reduced in patients with SVR after IFN-based therapy compared to non-responder (4.2% *vs* 17.8%; HR = 0.23)[161]. The majority of the studies report that older ages, male gender, advanced liver FIB, fatty liver disease, and a high post-treatment serum AFP level are the main risk factors for HCC development, even after SVR[162].

DAAs

The perspective of HCV therapy has changed over the years after the introduction of DAAs therapy, which has increased the SVR rate up to 90%, with excellent tolerance compared to IFN-based regimens. Even if the therapies demonstrated high efficacy, two retrospective studies, published in 2016 and conducted respectively in Spain and Italy, supposed the existence of a higher risk of HCC occurrence and recurrence following DAAs treatment course, raising the debate about the safety profile of DAAs and, in particular, the relation between SVR with and HCC occurrence and recurrence[163]. The Spanish study registered a high recurrence rate of HCC (27.6%) among 58 patients with previous history of HCC who received DAAs[164]. In the Italian study, HCC was detected in 7.6% of patients following DAAs treatment, 28.81% of patients with and 3.16% of patients without previous HCC[165]. Furthermore, an unpredictable increase in the amount of advanced stage HCC was noticed by the same authors.

Later debates arose owing some possible biases in these studies (the small size of the cohorts, lack of untreated controls, and short follow-up periods). Subsequently, further

data from large real-world retrospective cohort studies with extended follow-up periods after DAAs treatment have been released. On the heels of the studies listed so far, in 2017 another meta-analysis conducted by Waziry *et al*[166] reported lack of statistically significant increase in HCC recurrence or occurrences in either patients having undergone DAAs or IFN treatment after achieving SVR. Subsequently in 2018, a systematic review from Ioannou *et al*[167] including 62354 patients treated with DAAs, IFN or a combination of both, showed no significant difference in HCC occurrence or recurrence between the three treatment regimens. In fact, SVR was associated with a 71% decrease in HCC occurrence risk, with no particular differences between DAAs and IFNs. Nahon *et al*[168] enrolled 1270 HCV infected patients with compensated cirrhosis in France, demonstrating that there wasn't any statistically significant increase in the risk of HCC occurrence following DAAs treatment (HR = 0.89), after adjustment for age, diabetes and reduced liver function. In 2018, another American study from Singer *et al*[169] showed that DAAs therapy was associated with a lower risk of HCC as compared to untreated patients (HR = 0.84) and to IFN based treatment (HR = 0.69). During the same year, an Italian study found that SVR after DAAs treatment led to a lower incidence rate of HCC in patients with HCV-related cirrhosis over a follow up of 14 mo[170]. At the end of 2018, another Italian review from Guarino *et al*[171] outlined that the alarmist data on HCC recurrence after DAAs treatment and more aggressive pattern of disease have not been confirmed by subsequent studies.

Recently, a French prospective cohort study on 9895 HCV infected patients with a long term follow-up (mean 33.4 mo) reported that, after adjusting for several variables (including non modifiable risk factor as age, sex, geographical origin, HCV genotype and modifiable ones as FIB score, alcohol consumption, diabetes, arterial hypertension, and model for end-stage liver disease score in patients with cirrhosis), DAAs exposure decreased all-cause mortality (adjusted HR = 0.48) and the risk of HCC development (adjusted HR = 0.66)[172]. Another large American retrospective cohort study concluded that DAAs treatment and SVR resulted in a significant benefit in overall mortality[173].

Watanabe et al[174] recognized FIB-4 index ≥ 4.0 and albumin ≤ 3.8 g/dL before DAAs therapy and a FIB-4 index ≥ 4.0 and AFP ≥ 6.0 after the end of DAAs therapy as independent predictors for HCC occurrence. Another study confirmed a linkage between the risk of HCC and persistently high FIB-4/[aminotransferase/platelet ratio index (APRI)], either with or without cirrhosis. HCC development risk shows a significant reduction in cirrhotic patients who showed a decrease of FIB-4/APRI scores after DAAs therapy[175].

In 2020, an Egyptian study designed with three large independent cohorts, enrolled 4400 patients with chronic hepatitis C and advanced FIB, and identified predictors of HCC development after DAAs treatment developing and validating a simple risk score (GES risk score). GES identifies three groups of patients with a different risk of HCC occurrence along the follow-up, by means of simple and readily available predictors: Age, male gender, low albumin, high AFP levels and presence of cirrhosis at baseline. The cumulative incidence of HCC for GES low-risk group at two years was 1.2% in the derivation cohort and 0.22% in validation cohorts, while GES high-risk group showed a 6 to 30 folds higher risk, with a cumulative incidence of 7.1% and 6.1% in the derivation and validation cohorts, respectively. The study enrolled mostly genotype 4 infected patients, thus further studies are needed to assess the utility of this score in clinical practice[176].

These data underline that the rise in HCC incidence suggested by the first studies in patients with HCV-related cirrhosis treated with DAAs who achieve SVR compared to those treated with IFN-based therapy may be due to confounders as patients features (age, diabetes, reduced liver function) and/or lower screening frequency. Therefore, DAAs treatment should not be withheld for the potential risk of HCC occurrence or recurrence. According to long-term post-SVR observational studies, HCC development risk remains high in patients with cirrhosis who eliminate HCV, although it is significantly reduced compared to untreated patients or patients not achieving SVR (10-year cumulative incidence rate 21.8% vs 5.1%)[139].

CLINICAL PRESENTATION OF HBV AND HCV-RELATED HCC: A COMPARISON

Several studies assessed the difference in clinical manifestation in HCC patients between different etiologies. Different HCC surveillance strategies need to be set according to the viral etiologies, in relation with HCC age of presentation and clinical features. Two Italian studies noticed that HBV-infected patients are younger at HCC diagnosis, with a higher staging, increased probability of vascular invasion, and a worse prognosis[177]. An Asian study showed a peak incidence of HBV-related HCC in the 50-59 year old group, whereas the peak annual incidence in HCV patients is over 70 years. In an HBV high endemic area, the outcome analysis of the study showed a reduced survival in HBV-related HCC compared to HCV-related HCC (1.34 *vs* 2.17 years, adjusted for confounders). There was also a slight increase in frequency of large tumors (> 5 cm) and portal vein invasion at diagnosis in HBV-related HCC group, while multiple tumors occurred more frequently in HCV patients[178]. A cross sectional observational study from Pakistan, an HCV high endemic country, confirm that HBV-related HCC patients were younger at presentation, but HCV-related HCC was larger, with higher AFP serum levels and more frequent vascular invasion at diagnosis[179]. Similar results were obtained in a large cohort including Asian and European patients[196,199]. These differences can be explained by the high prevalence of perinatal infections in high endemic areas and to the specific oncogenic potential of HBV. However, there was no difference in post-operative or post-treatment outcomes in the studies listed above between HBV and HCV-related HCC groups.

CONCLUSION

Chronic HBV- and HCV-related hepatitis are still pandemic, with an increased spread in low and middle-income continents, such as Asia and Africa. HBV and HCV infected patients have a higher oncogenic risk compared to other liver disease aetiology, mostly because of the interactions between viral proteome and transcriptome and host cells molecular pathways, involving inflammation and cells replication. New data about gut-

liver axis and systemic inflammation are pointing out their key role as a cofactor in hepatocarcinogenesis. Therefore, viral hepatitis B and C still represent a major public health problem worldwide, although health organizations have made substantial efforts to contain their spread in recent years, through the global HBV vaccination campaign and the widespread accessibility of DAAs for HCV. The broad availability of generic antivirals would allow low- and middle-income countries to strengthen their contribution to the HCV eradication program. In the meantime, one of the most important challenges is to adapt the timing of HCC surveillance in these patients, with the aim of ensuring early diagnosis and rapid management to improve prognosis. In the era of coronavirus disease 2019, well-organized and accessible HCC surveillance becomes even more important due to the limited healthcare resources allocated to secondary prevention and the reluctance of patients to access healthcare facilities. In the future, new biomarkers and risk stratification systems may help clinicians to identify patients for closer follow-up, leading to a more personalized approach to HCC surveillance.

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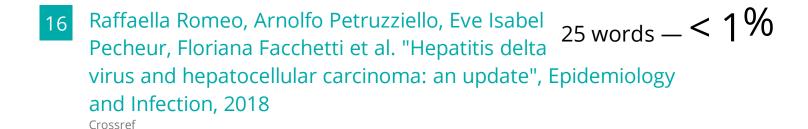   Internet   35 words — < 1%

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