

2016 Hepatocellular Carcinoma: Global view

Significance of hepatitis virus infection in the oncogenic initiation of hepatocellular carcinoma

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Author contributions: Sukowati CHC designed the review scheme and wrote the manuscript; El-Khobar KE, Ie SI and Anfuso B wrote the manuscript; Muljono DH and Tiribelli C provided idea and feedback of the manuscript; all authors read and approved the manuscript.

Conflict-of-interest statement: All authors have no conflicts of interest.

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Received: May 28, 2015
Peer-review started: June 1, 2015
First decision: July 14, 2015

Revised: August 6, 2015

Accepted: October 12, 2015

Article in press: October 13, 2015

Published online: January 28, 2016

Abstract

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related death worldwide. Chronic infection of hepatitis B virus (HBV) and/or hepatitis C virus (HCV) is a major risk factor in the development of the HCC, independently from excessive alcohol abuse and metabolic disease. Since the biology of HBV and HCV is different, their oncogenic effect may go through different mechanisms, direct and/or indirect. Viral hepatitis infection is associated with cellular inflammation, oxidative stress, and DNA damage, that may lead to subsequent hepatic injuries such as chronic hepatitis, fibrosis, cirrhosis, and finally HCC. Direct oncogenic properties of these viruses are related with their genotypic characteristics and the ability of viral proteins to interact with host proteins, thus altering the molecular pathways balance of the cells. In addition, the integration of HBV DNA, especially the gene *S* and *X*, in a particular site of the host genome can disrupt chromosomal stability and may activate various oncogenic mechanisms, including those in hematopoietic cells. Recently, several studies also had demonstrated that viral hepatitis could trigger the population of hepatic cancer stem cells. This review summarize available pre-clinical and clinical data in literature regarding oncogenic properties of HBV and HCV in the early initiation of HCC.

Key words: Hepatocellular carcinoma; Hepatitis B virus; Hepatitis C virus; Oncogenicity; Viral pathogenicity

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Core tip: According to the most recent data released by the International Agency for Research on Cancer-World Health Organization, liver cancer is the second most common cause of cancer mortality worldwide. Hepatocellular carcinoma (HCC) accounts for around 90% of liver cancer cases and it is variably distributed according to its main risk factors. The hepatotropic viral [hepatitis B virus (HBV) and hepatitis C virus (HCV)] infection can cause a disarrangement in cellular pathways through an indirect and/or direct mechanism in liver injury. This review summarize available data in literature regarding the oncogenic properties of HBV and HCV in the initiation of HCC, including their role in the activation of hepatic stem cells.

Sukowati CHC, El-Khobar KE, Ie SI, Anfuso B, Muljono DH, Tiribelli C. Significance of hepatitis virus infection in the oncogenic initiation of hepatocellular carcinoma. *World J Gastroenterol* 2016; 22(4): 1497-1512 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i4/1497.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i4.1497>

HEPATOCELLULAR CARCINOMA: EPIDEMIOLOGY AND RISK FACTORS

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancers, it accounts for around 90% of all cases^[1]. According to the Globocan 2012 data of the International Agency for Research on Cancer-World Health Organization, it is the fifth most common cancer in men and the ninth in women, and the second most common cause of cancer-related death, estimated to be responsible for around 9% of all cases in 2012^[2-4].

The global distribution of HCC is associated with the prevalence of its dominant risk factors. Infection of endemic hepatitis B virus (HBV) is the major cause of HCC in eastern Asia and sub-Saharan Africa for around 70%. In Europe and North America countries, hepatitis C virus (HCV) infection ranges from 50%-70% while excessive alcohol consumption leading to alcohol steatohepatitis (ASH) contributes for around 20% of all cases^[1,5,6]. In its development, HCC usually emerges from a long-term chronic disease course with underlying liver cirrhosis (around 80%)^[7]. However, it should be noted that HCC can occur in non-cirrhotic liver, accounts for around 20% of all cases^[8].

Besides the infection of hepatotropic viruses and alcohol, obesity and diabetes that commonly associated with non-alcoholic steatohepatitis (NASH) increase the risk of HCC. Synergism between hepatitis virus infection and metabolic liver disease seems to worsen the course of the disease. Certain toxins and chemical agents such as aflatoxin B1 and vinyl chloride monomer also contribute in the progression of HCC. The cumulative risk for HCC is higher in male

gender compared to female^[3,9,10]. It has been thought to occur because of life style, male is more prone to viral infection and alcoholic cirrhosis. However, it is important to note that hormones (testosterone, progesterone, and estrogens) also take part in viral infection and subsequent liver damages^[10].

HCC is a heterogeneous disease with various features and prognostic types. HCC is commonly developed in an extended period and different treatment options may vary between individuals. Based on the consensus of the Barcelona Clinic Liver Cancer (BCLC) staging system, liver resection is the best treatment option for very early stage HCC (0), liver transplantation and radiofrequency or percutaneous ethanol injection (PEI) for early stage (A), trans-arterial chemoembolization (TACE) for intermediate stage (B), molecular treatment with Sorafenib for advanced stage (C), and supportive palliative care for terminal stage (D)^[11]. Based on continental and geographical policy, Fong and Tanabe compared the guidelines in Asia, Europe, and America, and they showed that most guidelines are similar with some variances in disease surveillance and treatment allocation recommendations^[12].

It has been widely known that hepatocarcinogenesis is accompanied with complex aberrations in developmental and oncogenic molecular signaling pathways (excellently reviewed by^[13,14]). Until now, a number of molecule-targeted drugs has been developed or under development to treat patients that cannot receive curative intervention. Various novel and promising drugs have been developed and tested in various phases of clinical trials, as a single agent or combined regimen^[15]. As mentioned above, Sorafenib is the approved drug for the treatment of advanced HCC. Sorafenib is a multikinase inhibitor agent that targets the molecular pathway alteration commonly observed in HCC, including that of Wnt- β catenin pathway^[16,17].

HEPATITIS VIRUS INFECTION

HBV infection

HBV, a member of *Hepadnaviridae* family, is a partially double-stranded DNA virus with 3.2 kb genome size. HBV genome contains 4 major open reading frames (ORF) that encode for polymerase (pol) for reverse transcriptase activity and replication, surface protein (HBsAg), core that form nucleocapsid and secreted HBeAg, and X that is important in viral replication^[18,19]. Hepatitis B is one of the major public health problems that affect approximately 2 billion people globally, with more than 240 million chronic carriers and more than 780000 deaths annually^[20]. By 2010, half of HCC cases are HBV-related, with or without history of liver fibrosis^[4,21,22].

Based on several studies, HBV-related HCC development is mainly associated with risk factors such as male gender, persistently high HBV DNA levels,

hepatitis B e antigen (HBeAg) positivity, presence of liver cirrhosis, older age, persistently high ALT levels, family history of HCC or chronic infection from perinatal transmission, and co-infection with HIV and/or HCV^[23-28]. For example, male gender has hazard ratio (HR) 2-8 times more for HCC development compared to female^[23-26]. Similarly, higher HBV DNA levels is associated with higher incidence of HCC compared to HBV DNA levels lower than 10000 copies/mL^[24]. HBeAg positivity and ALT levels ≥ 45 U/L have HR 4.3 and 4.1, respectively, while liver cirrhosis is associated with 10.8-33.3 increased risk of HCC development compared to chronic hepatitis B patients without cirrhosis^[24,25,29]. Based on these analyses, algorithms to screen and monitor high-risk populations have been proposed in many guidelines, which may reduce the incidence of HCC-related mortality because of the poor prognosis of advanced HCC development^[30-33].

Prevalence of HBV has been shown to be reduced with the introduction of hepatitis B immunization program in newborns, complemented with administration of hepatitis B immunoglobulin for those born to mothers with chronic HBV infection^[34-36]. Due to the commitment to eradicate the vertical transmission of HBV through national mass vaccination policy, the prevalence of hepatitis B can be decreased that leads to reduction of HCC cases, as demonstrated in the successful national program in Taiwan^[37,38]. Prevention of HCC development by HBV vaccination is in line with the new Sustained Development Goals proposed by WHO, in which HBV-related HCC is one of the three preventable cancers that make up the bulk of cancer-related mortality globally^[22].

HBV genotype

HBV naturally-occurring genetic variations such as genotypes and subgenotypes, as well as mutations in some of the HBV genomic regions have been associated with different clinical manifestations such as development of cirrhosis and/or HCC^[27,39]. Currently, HBV is classified into 9 genotypes (A to I) and one putative genotype (J) based on genome-wide divergence of more than 7.5%^[40,41]. The distribution of the different HBV genotypes is geographically-related, most likely in association with the distribution of the different ethnic populations worldwide^[42-45].

Since hepatitis B is endemic mainly in the Asia Pacific regions with HBV genotype B and/or C domination, most reports on the relation between genotype and HCC development concerns these two genotypes. Most reports propose HBV genotype B to be more lenient than genotype C, with some exceptions^[39,46,47]. In general, HBV genotype C is commonly associated with later HBe seroconversion, more severe liver diseases, as well as faster progression of liver fibrosis and HCC development, although the life-long risk remain similar between genotype B and C^[46-50]. In addition, HBV genotype

B is associated with better response to treatment, enhancing the prognosis and reducing the risk of advanced disease progression^[45,51]. Even compared to other genotypes, genotype C appeared to have worse prognosis in term of severe advanced liver disease development, with HR 2.05-2.34 times more than HBV genotype B or A and D, the four major HBV genotypes associated with HCC development^[52]. This might be because HBV genotype C has higher tendency to induce DNA double-strand breaks and accumulate reactive oxygen species (ROS) that causes endoplasmic reticulum (ER) stress, in addition to more efficient cellular homologous-recombination events that increase the risk of chromosomal rearrangements and DNA damage, stimulating the formation and development of HCC^[53]. Thus, genotyping of HBV is an important diagnostic tool in predicting the prognosis and response of therapy in hepatitis B patients.

HBV genetic mutation

Based on many reports, a double mutation in the basal core promoter (BCP) region of HBV genome (A1762T/G1764A) is associated with 1.7-10.6 fold increased risk of HCC incidence, particularly for those infected with HBV genotype C compared to genotype B^[46,54-57]. In addition, in combination with C1653T and T1753V point mutations, these BCP mutations are associated with increased risk of HCC in HBeAg-positive than HBeAg-negative subjects, which can predict up to 80% of HCC development^[24,46]. On the other hand, precore G1896A mutation is associated with HBeAg-negativity, but not with increased risk of liver cirrhosis or HCC development^[46,54,55]. A report on novel mutation in genotype D observed the significance of T1858C mutation in the precore region that is associated with HCC progression in HBeAg-negative sample^[53]. This report also identified several amino acid changes in HBV core antigen (HBcAg) that is associated with HCC development, namely I116L, P130Q, and T147C; two of which are parts of B- and killer T-cell epitopes^[58].

Mutations in other regions of HBV genome have also been linked with disease progression, especially the PreS region. Mutations or deletions in the PreS region accumulated with the progression of chronic hepatitis B, and were associated with significant increase of HCC risk, even in adolescent^[46,59]. An amino acid change S98T in the PreS1 region showed significant association with progression of liver fibrosis to cirrhosis and HCC, particularly in HBeAg-negative patients^[58].

The accumulation of HBsAg particle in the ER of hepatocytes leads to the histological appearance of ground glass hepatocytes (GGH)^[60]. It was repeatedly shown that hepatocytes expressing various forms of PreS2 mutants often developed into type II GGH, which exhibited ER and oxidative stress due to protein retention and DNA damage, while simultaneously created genomic instability and up-regulation of cell

cycle progression and proliferation^[61-63]. A mutation in codon 38 of X gene was preferentially found in patients with HCC and can be used as an independent risk factor for the development of HCC^[64]. These newly defined oncoproteins may be utilized to develop novel biomarkers to predict HCC development^[62].

Co-infection HBV/HDV

Hepatitis D virus (HDV) is an imperfect RNA virus which needs HBV to be able to replicate^[65]. Therefore, HDV always presents as co-infection or super-infection in approximately 5% of HBV-infected individuals, and causes more severe outcome such as fulminant hepatitis, liver cirrhosis, and HCC^[65-67]. Super-infection often manifests in a rapidly progressive disease leading to cirrhosis within 2 years in 10%-15% of patients^[68]. HBV/HDV co-infected patients usually have higher ALT and bilirubin levels as well as a higher prevalence of liver cirrhosis and HCC^[69]. HDV co-infection is considered as a risk factor for HCC (HR 1.4-6.0 fold compared to HBV mono-infection), with lower survival rate^[56,70-73]. In addition, HDV co-infection significantly increases the incidence rate of other gastrointestinal-related diseases in enhanced magnitude, and mortality rate of severe hepatitis manifestations^[74]. Recently, it has been reported that high serum level of HDV RNA can be used as a predictor of cirrhosis and liver cancer in patients with chronic HDV infection^[75]. Interestingly, although HBV/HDV co-infection leads to faster cirrhosis or HCC development in immuno-competent individuals, it may actually take part in lengthening the survival of liver-transplant or graft patients that are immuno-compromised, even though the mechanism is still unclear^[76].

Recent advances in the development of highly effective HBV vaccine can be used as a preventive measure not only in the reduction of HBV infection, but also in the decrease of HDV, its associated pathogen^[77]. The development of animal model with chronic hepatitis delta infection^[78] can be utilized as a tool to study its pathogenesis *per se* and to discover its significance in the development of HCC. Detailed aspect on HDV viral biology, epidemiology, pathogenesis, and treatment is reviewed in^[79,80].

HCV infection

HCV, a member of *Flaviviridae* family, is a single stranded RNA virus with 9.6 kb genome size. HCV genome is processed into structural proteins core, E1, and E2, and non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B^[18]. Chronic HCV infection affects approximately 170 million people worldwide, and may lead to development of liver fibrosis, cirrhosis, and HCC^[81,82]. HCV infection may result in extra hepatic manifestations and metabolic disorder, including insulin resistance, type 2 diabetes and cardiovascular disease^[83,84]. The rapidly evolving HCV treatment in the last

decade has caused a decline in the incidence rate of viral infection^[85]. However, the disease burden of HCV-related liver diseases is predicted to continue increasing in the approaching years^[86]. HCV life-cycle occurs mostly in the cytoplasm, with the viral replication complex enclosed within a membranous web structure that is closely associated with the ER membrane, mitochondrial outer membrane, and lipid droplets^[87,88].

HCV genotype

HCV is highly heterogeneous and can be classified into seven recognized genotypes (genotype 1 to 7) and multiple subtypes based on the differences of the whole viral genome. Genotypes and subtypes can be divided into quasispecies based on genetic diversity^[89]. HCV genotypes have different susceptibility to interferon (IFN), thus HCV genotyping is used to determine the type and duration of antiviral therapy^[90]. Treatment with IFN-based regimen resulted in high sustained virological response (SVR) (about 80%) in genotype 2 and 3-infected patients, while genotypes 1 and 4 have lower SVR (about 50%) and genotypes 5 and 6 have intermediate response rates^[91,92].

HCV genotypes have been associated with distinct pathological features, such as liver steatosis, insulin resistance, inflammation, and hepatitis reactivation^[93-98]. In regards on the association between HCV genotype and risk of developing HCC, the available evidences are quite inconsistent^[10]. Early study showed that genotype 1b patients have a significantly higher risk of developing HCC^[99]. This early observation was supported by the result of a seventeen-year prospective cohort study, which showed 44 out of 104 genotype 1b followed-up patients developed HCC^[100]. A meta-analysis study that calculated age-adjusted risk estimated genotype 1b patients had almost double the risk of developing HCC in comparison with patients infected with other genotypes^[101]. On the other hand, recent studies suggest the association between genotype 3 and accelerated liver disease progression^[102]. A retrospective cohort study involving 353 patients showed that genotype 3 patients develop more HCC compared to non-3 patients (44%-26%)^[103]. These data were confirmed by successive studies with large cohort of infected patients that showed, even after adjustment of age, clinical, and antiviral treatment factors, genotype 3 had a higher risk of developing cirrhosis and HCC than genotype 1 patients^[104,105].

ONCOGENICITY OF HEPATITIS VIRUS

HBV X protein

Chronic infection of HBV and/or HCV as a risk factor in the development of HCC is clearly acknowledged, as shown in Figure 1. Advances in *in vitro* technique and transgenic animal model with the insertion and

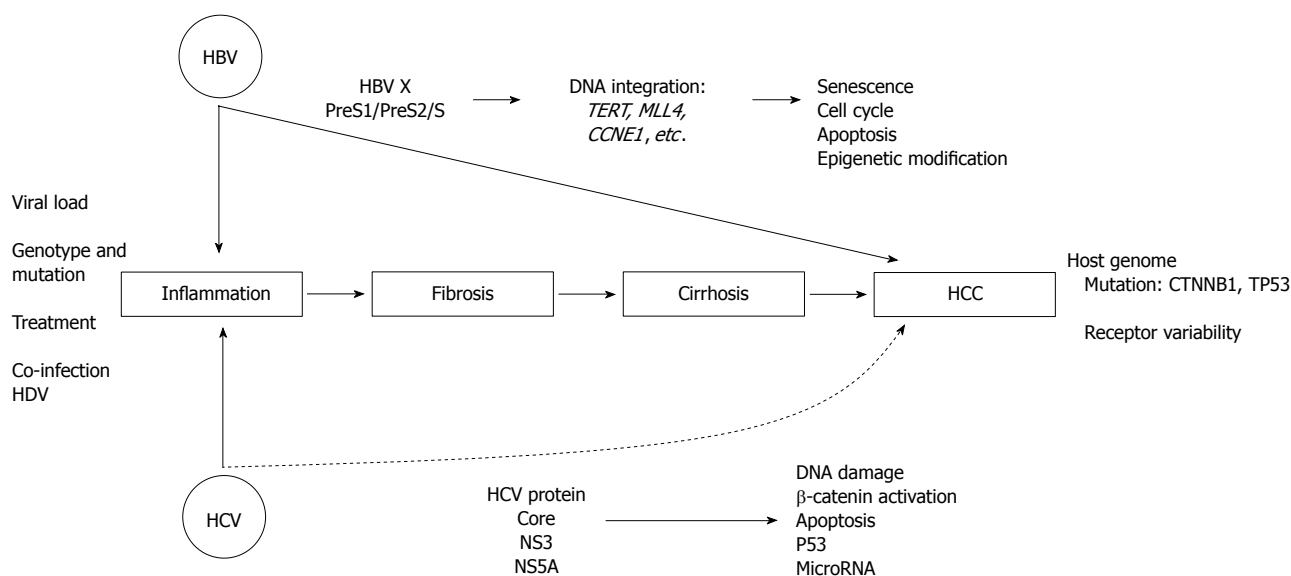


Figure 1 Oncogenicity of hepatitis B virus and hepatitis C virus in hepatocarcinogenesis. HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma.

modification of viral transgene^[106] have opened many possibilities to understand the involvement of viral proteins in cellular damage. However, their pathogenesis in the oncogenic initiation of hepatocarcinogenesis is still unclear. Limitation in the cellular biology, for example a low efficiency in viral replicon *in vitro* hampers the study of the pathogenicity of these agents.

HBV infection causes immunological response that may lead to oxidative stress and successive DNA damages of the cells (reviewed in^[107]). Direct oncogenic property of HBV sequence by integration of its DNA into human genome can explain the incidence of non-cirrhotic HCC^[108]. This insertion might involve deletions, cis/trans-activation, translocation, production of fusion transcripts and generalized genomic instability^[109,110]. Consequently, it may lead to disruption of the host cellular pathway. Nevertheless, non-cirrhotic HCC with low grade fibrosis can be found also in HCV and NASH-related HCC^[111,112], probably through a different oncogenic cascade. HBV DNA integration is present in majority of HBV-related HCC, even though it is also found in non-tumor tissue and chronic hepatitis B without HCC^[113,114]. HBV DNA integration is considered as a strong oncogenic effect in hepatocarcinogenesis even though a recent study has proposed a contrasting evidence^[115].

Direct oncogenic property of HBV, in particular for HBx and surface protein HBs has been intensely investigated. HBx is composed of 154 amino acids with a molecular mass of approximately 17.5 kDa. It has pleiotropic functions as an important regulator in viral life cycle, a transcriptional activator, and a stimulator in the cytoplasmic signal transduction pathways^[116]. Expression of HBx protein was found to be present in

around 50%-60% of HBV-infected liver cirrhotic and HCC tissues, noticed in the cytoplasm of parenchymal and neoplastic cells^[117].

The presence of HBx has been associated with tumor suppressor p53 rescue of apoptosis^[118]. A European study showed p53 is a frequent altered pathway in HBV-related HCC. Mutations of host TP53 was associated with shorter survival and interestingly it was also related with genotype B of the patients from Asia and sub-Saharan Africa^[119]. The interaction between phosphorylated Ser41-Pro motif of HBx and peptidyl-prolyl isomerase Pin1, a regulator of p53, followed by cis-trans isomerization and stabilization, significantly augmented the expression of HBx downstream target genes, leading to oncogenesis^[120]. Based on this study, it was revealed that Ser41-Pro motif was conserved in HBV genotype A and B, but altered in most of the genotype C. Further analysis of eight HBV genotypes A-H showed a correlation between this motif and HBV genotypes, associated with HCC distribution and age^[121].

Integration of HBV X sequence into host genome is a common event in HCC^[113]. It was reported that HBV X integration occurred more often in HCC than in cirrhosis and it was positively related with the level of cell-cycle and apoptotic protein, including Cyclin A, retinoblastoma protein (Rb), Fas-associated death domain protein (FADD), tumor necrosis factor receptor-associated death domain protein (TRADD), and nuclear factor kappa B (NF- κ B)^[122]. Whole genome sequencing of HCC samples have demonstrated HBV X DNA integration within or upstream the sequence of telomerase reverse transcriptase (*TERT*), epigenetic regulator MLL4, and cell cycle gene *CCNE1* as "hot site" breakpoints^[123-125]. DNA integration was frequently

observed in the tumors (around 85%) compared to adjacent liver tissues (around 30%)^[125]. Large numbers of HBV integration (defined as ≥ 3) was positively associated with the serum level of HBsAg and AFP, and importantly, with low survival time compared to those with no or low numbers of integration^[125]. HBx is associated with epigenetic modifications through interaction with histone deacetylase 1 and DNA methyltransferase during hepatocarcinogenesis^[126-128].

A recent article reported development of HCC in the absence of severe liver damage in a HFE-haemochromatosis patient that was seronegative for hepatitis B and C infections. HBx gene sequence was detected in tumor but not in non-tumor. HBV integration involved a 5'-deleted X gene with an intact enhancer- Π /basal-core promoter region and integrated upstream of the partitioning-defective-6-homolog-gamma gene (PARD6G)^[129]. The role of HBV X gene in hepatocarcinogenesis is reviewed in^[130,131].

HBV S protein

ORF S gene region with three translational start sites PreS1, PreS2, and S, encodes for large (L), middle (S) and small (s) surface protein (HBs), respectively. S protein is composed of 226 amino acids, M protein is S with additional 55 amino acids, and L protein is M with additional 108 or 119 amino acids, based on virus genotype^[132,133]. S region is conserved while both PreS1 and PreS2 are variable and prone to genetic mutations. Besides genotyping based on whole genome sequence, the variation in PreS2 region has been used to determine HBV subgenotypes^[134]. As mentioned previously, different GGH appearances showed different mutated S proteins. Several studies from the group of Su *et al.*^[61-63] had shown that type I GGH harbored mutants with deletions within the pre-S1 region while type II GGH contained PreS2 mutants^[61-63]. In hepatocarcinogenesis, PreS2 mutant was demonstrated to produce an aberrant Cyclin A expression and centrosome over-duplication through ER stress that led to chromosomal instability^[135].

A transgenic animal model with the insertion of PreS/S gene regions expressed high level of HBsAg, showed inflammation and appearance of GGH, preneoplastic lesion, and finally it led to HCC in major number of animals^[136], indicating a direct oncogenic input of this gene. Gene expression profile of 3-month old mice showed differentially expressed genes involved in various regulations such as apoptosis, cell cycle, NF- κ B signal transduction pathway, and inflammatory response^[137].

As that of HBV X DNA, the insertion of S regions into host genome has been widely reported, first noticed in the WHV-infected animals. In their study, Sung *et al.*^[125] observed recurrent integration of PreS1, PreS2, and S sequences in human genes TERT and MLL4, even though they did not notice it in CCNE1 gene as HBV X sequence.

A recent study reported that in HCC patients with occult hepatitis B, HBV DNA integration was found in around 75% of cases, in which the inserted viral genes were mainly X and PreS/S, followed by C and Polymerase sequences^[138]. Furthermore, in a prospective 12-years study in chronic hepatitis C patients with occult hepatitis B, X integration can be associated with HCC development in the absence of cirrhosis^[139]. The HBV DNA integration is not only observed in liver cells but also in blood cells. The transcript of HBsAg coding gene and the integration of HBV DNA in bone marrow haematopoietic stem cells from chronic HBV infection patients was observed^[140].

HCV and oxidative stress

Since HCV RNA cannot integrate into human genome, at the beginning, the mechanism in HCV-related HCC pathogenesis is thought majorly to be indirect pathways *via* the effects of chronic inflammation and oxidative stress. Subsequently, it leads to fibrosis and eventually cirrhosis as observed in the other HCC etiologies such as ASH, NASH, and obesity-related disorder. However, current literatures also showed a direct oncogenic effect of the viral proteins^[141].

Oxidative stress has been implicated as one of the mechanisms of HCV-induced hepatocarcinogenesis^[142]. Oxidative stress occurs when there is imbalance in the production and clearance of ROS. ROS is a normal by-product of numerous cell processes including proliferation, apoptosis, and cell senescence^[143]. In the liver, ROS is mainly produced by mitochondria in hepatocytes, and from nicotinamide adenine dinucleotide phosphate oxidase and xanthine oxidase reactions in Kupffer cells and inflammatory cells^[144]. Long-term oxidative stress may induce DNA damage, and since ROS can also functions as second messenger in cellular signaling, increased ROS level may trigger the activation of oncogenic signaling pathways^[107,142].

Increased oxidative stress in chronic hepatitis C patients has been shown through elevated levels of several oxidative stress biomarkers, including 8-hydroxydeoxyguanosine (8-OHdG), malondialdehyde, and thioredoxin in both sera and liver biopsy samples^[145-147]. Chronic hepatitis C patients have also been shown to have higher expression of 8-OHdG, also an indicator for DNA damage, in comparison with chronic hepatitis B patients; suggesting that hepatic oxidative DNA damage is more common in chronic hepatitis C^[148]. Further, some clinical studies have shown that addition of antioxidant agent could improve oxidative stress-caused liver injury and maybe important for treatment management of HCV patients^[149,150].

HCV-effect on increased oxidative stress has been mainly attributed to the expression of viral core protein, although other viral proteins have also been shown to induce oxidative stress^[151]. The expression of HCV core protein, either *in vitro* or *in vivo*, have been

shown to induce alteration of mitochondrial function, increased ROS levels, and increased intrahepatic lipid peroxidation^[152-154]. HCV NS5A protein has been shown to alter intracellular calcium level and induces oxidative stress in an *in vitro* model^[155]. Increased ROS is linked to dysregulation of various cells signaling pathways, particularly those that regulate cell survival, apoptosis, and inflammation. Indeed, increased oxidative stress resulted in activation of p38 MAPK, JNK, NF- κ B and STAT3^[155-157], which promotes cell survival. Increased ROS have been linked to dysregulation of various cells signaling pathways, particularly those that regulate cell survival, apoptosis, and inflammation. Indeed, increased oxidative stress resulted in activation of p38 MAPK, JNK, NF- κ B and STAT3^[155-157], which promote cell survival. These activations induce subsequent activation of TGF β 1^[158], a major profibrogenic factor in the liver, causing activation of hepatic stellate cells^[159].

HCV core protein has also been shown to induce upregulation of TGF β 1 transcription, showing a more direct role of HCV protein in inducing fibrogenesis^[160]. Activated stellate cells could also produce TGF β 1 and other pro-inflammatory cytokines, which facilitate further inflammatory response^[161] and demonstrate close association between oxidative stress and inflammation in chronic hepatitis C. In addition, increased ROS production along with persistent viral expressions might also induced cell death, either through TNF α - or mitochondria-mediated apoptosis^[162,163]. The resulting apoptotic bodies will release alarming molecules that serve as pro-inflammatory mediators, further aggravating the oxidative stress-associated inflammation. Oxidative stress can also damage telomeres, causing them to shorten^[164] that has been reported occurring in the presence of increased oxidative stress marker 8-oHdG expression^[165], signifying the effect of HCV-induced oxidative stress on telomere shortening and senescence.

One of the consequences of chronic oxidative stress is oxidative DNA damage. ROS could interact directly with DNA to induce DNA damage^[107], and mitochondrial DNA (mtDNA) that has no protective histone protein is more susceptible to the damage^[144]. HCV infection has been reported to induce a mutator phenotype by causing dsDNA breaks^[166]. In line with this, decreased mtDNA in peripheral blood leukocytes of chronic hepatitis C patients have been reported, and the degree of DNA damage was found to be correlated with increased liver inflammation^[167] that may lead to progressively liver damage including HCC.

HCV and ER stress

The close association of the HCV viral replication complex to the ER membrane might cause ER dysfunction. Indeed, HCV has been shown to cause ER stress^[168,169]. ER stress occurs when ER function as the site of production and posttranslational modifications

of cell proteins is perturbed, which then triggered the unfolded protein response (UPR) pathway to restore protein homeostasis^[170,171]. This effect is achieved by initiating transmembrane protein ATF6 proteolytic cleavage^[169], subsequently activating the transcription of ER chaperone genes, GRP78^[172]. Furthermore, up-regulation of both GRP78 and transcription factor CHOP/GADD153 have been correlated with down-regulation of anti-apoptotic Bcl-2 gene expression, increased NF- κ B, and cleavage of caspase-3 and PARP^[173-175]. Increased GADD153 expression has also been linked to cell susceptibility to oxidant injury^[173], suggesting that ER stress and oxidative stress is closely related in pathogenesis of HCV-infection.

The expression of singular HCV proteins, particularly core, NS5A, and NS2 proteins and/or HCV subgenomic replicons in *in vitro* induced the UPR pathways^[169,174,176]. These findings were later confirmed using HCV transgenic mice model^[175,177]. The HCV-induced ER stress is reduced following treatment with interferon- α 2a treatment^[177] or NS3 protease inhibitor^[176], resolving UPR response and restoring protein homeostasis. ER is also major site for intracellular calcium storage, and these calcium ions are trafficked to and from the ER to regulate various cellular signal transduction^[171]. HCV core, and also NS5A, alters ER calcium homeostasis by inducing ER stress and depleting ER calcium content^[174,178], resulting in mitochondrial membrane depolarization and triggering mitochondria-mediated apoptosis. This HCV-core effect is fully diminished by restoring the ER calcium storage^[174]. The changes in calcium homeostasis in HCV-infected cells have been suggested as the result of viral-induced increase ROS production and oxidative stress^[178], again indicating a strong correlation between oxidative stress, ER stress, and mitochondrial dysfunction.

HCV direct oncogenicity

Recent literatures demonstrated that HCV protein core, NS3, NS4B, and NS5A, can induce cell transformation *in vitro* and *in vivo* mice transgenic model^[179]. The fact that HCV transgenic mice with the expression of a HCV viral protein can develop HCC, suggests a direct oncogenic effect rather than an inflammatory mechanism^[180]. Viral protein in cytoplasm has the ability to interact with host protein and to alter the stability of the cellular mechanism leading to carcinogenesis. Further, direct interactions between these viral proteins with numerous host cell factors have been shown to lead to dysregulation of wide range of cellular signaling, particularly those involved in cell proliferation, apoptosis, cell metabolism, immune responses and also oxidative stress^[179,181].

Core protein has been reported to directly deregulate the tumor suppressor p53 pathway based on viral protein over-expression cell culture system. The level of deregulation is unclear, since available reports have shown for both HCV-induced activation and repression of p53-dependent gene expression^[182].

Host genetic variation and somatic mutation in TP53, as well as CTNNB1 encoding β -catenin, was found to be significantly associated with young age and moderate and poor differentiated HCV-related HCC^[183]. CTNNB1 activating mutations were also found to be more frequent in HCV-related compared to HBV-related HCC^[119]. In an *in vitro* study, HCV core protein activated canonical Wnt signaling *via* regulations of several important molecules upstream of β -catenin and presumably resulted in promotion of cell proliferation^[184].

Recent report showed that expression of HCV protein also increased proto-oncogene c-Myc expression *in vivo* and in infected human livers. This change is mediated through Akt-dependent activation of β -catenin and might further contribute to HCV-related oxidative stress and genetic damage^[185]. Furthermore, it had been demonstrated that the expression of NS5A stabilized and accumulated β -catenin through the phosphorylation and inactivation of GSK3 β ^[186,187].

HCV NS5B RNA-dependent RNA polymerase forms a cytoplasmic complex with Rb, downregulating the Rb expression and its DNA damage responses^[188,189]. HCV core has also been reported to inhibit Rb expression^[190]. HCV NS3/4A protein directly interacts with ataxia telangiectasia mutated kinase (ATM); a tumor suppressor protein that detects dsDNA breaks, resulting in impaired DNA repair^[191]. In addition, HCV core also binds to NBS1 protein to inhibit Mre11/NBS1/Rad50 defective DNA-sensing complex, resulting in impaired ATM activation and inhibition of repair enzymes DNA binding^[192]. HCV impairs the expression of NEIL1 DNA-excision glycosylases, as shown *in vitro* and in liver biopsy specimens of advanced liver disease patients^[193]. These observations suggest that HCV induces accumulation of DNA damage by inhibiting multiple DNA repair processes and promoting chromosome instability with consequent malignant transformation.

The association between HCV viral proteins with several tumor suppressor genes as listed above might affect the regulation of cellular senescence. Senescence pathway responds to cellular stress and acts to limit the proliferation of damaged cells^[194]. Inflammation, oxidative, and oncogenic stress can induce premature senescence, and this change is characterized by cell-cycle arrest, resistance to apoptosis, and oncogenic epigenetic changes^[142,194].

Recent studies had implied the role of several microRNAs in HCV-related HCC. Several studies had shown that miR-122, a liver-specific microRNA, was down-regulated in the majority of HCC samples analyzed, apart from HCV-related HCC (reviewed by Borel *et al.*^[195]). Previously it had been shown that the miR-122 regulated cell cycle protein Cyclin G1 that affected the stability of p53 and also altered chemotherapy sensitivity^[196]. The involvement of miR-122 in HCV-induced hepatocarcinogenesis is

reviewed in^[182].

ACTIVATION OF HEPATIC STEM CELLS

Accumulating evidences highlight the importance of cancer stem cells (CSC) in HCC biology. Different hepatic CSCs have been reported in various subtypes of HCC and they are considered as the master regulators of HCC initiation, progression, and metastasis^[197,198]. By using immunostaining and RNA-FISH for stem cells markers OV6, CK19, and CD133, the frequency of positive stem cell markers in liver cirrhosis and HCC roughly correlated with the relatively frequency of HCC that develops in the clinical setting^[199].

Despite the increasing importance of this heterogeneous population in driving carcinogenesis, little is known about the effect of viral hepatitis in the biology of CSC. Histological analysis of human tissue found a positive correlation between HBV infection and CD90^[200] and an inverse correlation with CD133^[201]. However, since co-staining of the CSC markers and the HBV proteins was not performed, is not clear if and how HBV alters the physiology of CD90+ and CD133+ CSC. Furthermore, it will be also important to put virus genotype in consideration to assess the role of a specific virus type with the phenotype of the cells.

Several reports have described the involvement of HBV in the generation of CSC. In particular, a correlation between HBx expression and EpCAM+ CSC appearance was clearly demonstrated since Arzumanyan *et al.*^[202] showed that the pluripotent stem cell transcription factors Oct-4, Nanog, and Klf-4, as well as EpCAM and β -catenin, were up-regulated in HBx expressing cells. Phenotypically, HBx stimulated cell migration, growth in soft agar, and spheroid formation. These data were confirmed in HBx transgenic mice fed with 3,5-diethoxycarbonyl-1,4-dihydrocollidine where an elevated number of EpCAM+ cells with characteristics of human progenitor cells was observed^[203]. Transformation of rat oval cells with HBx and the subsequent injection in nude mice treated with aflatoxin B1 *in vivo*, gave rise to tumor that expressed markers of adult hepatocytes as albumin and CK18, undifferentiated marker AFP, and oncoprotein c-Myc^[204].

Moreover, clinical evidence showed that high HBx expression in human HBV-related HCC was statistically associated with expansion of EpCAM+ or OV6+ tumor cells, aggressive clinicopathological features^[203,205], activated β -catenin signalling, and up-regulation of miR-181^[202]. In 2015, Fan *et al.*^[206] investigated the molecular mechanism by which HBx induces EpCAM expression, suggesting DNA demethylation as major mechanism driving the re-expression of EpCAM into hepatocytes.

Infection of HCV has also been associated in the induction of CSC, perhaps in a direct oncogenesis manner. The expression of an HCV subgenomic replicon in cultured cells resulted in the acquisition of CSC traits including an enhanced expression of

doublecortin and CaM kinase-like-1, Lgr5, CD133, AFP, cytokeratin-19, Lin28, and c-Myc. Conversely, curing of the replicon from these cells results in diminished expression of these factors. The analysis of liver tissues from HCV-positive patients and liver tissue microarrays reiterated these observations^[207].

The effect of the HCV nonstructural NS5A protein was studied in a transgenic mouse model. Viral protein, in synergy with alcohol-induced endotoxemia, induced the up-regulation of the Toll-like receptor 4 (TLR4) with the consequent expression of the pluripotency gene Nanog, a downstream gene up-regulated by TLR4, and CD133^[208]. On the other side, the CD133+/CD49f+ cells isolated from HCC developed in HCV core transgenic mice were tumorigenic both *in vitro* and *in vivo* and the TLR4-Nanog pathway was necessary for the maintenance of tumorigenic properties^[209].

FUTURE PERSPECTIVE

Collectively, literature review had demonstrated the significances of hepatotropic HBV and HCV during hepatocarcinogenesis. In the oncogenic initiation, they can induce immunological responses lead to successive damages of the liver cells that may direct the development of HCC. Since the biology of HBV and HCV is different, their oncogenic effect may go through a different mechanism, direct and/or indirect, as had been demonstrated in many pre-clinical and clinical studies. Even though studies in *in vitro* and transgenic animal model had expanded the knowledge of viral-specific proteins, the mechanism of the viral particle in inducing hepatocarcinogenesis is still unclear and open for discussion. This is partially due to several methodological limitations such as the difficulty on viral culture, and transgenic animal model cannot reflect the entire virus particle and its interaction with host cells receptor (*e.g.*, hepatocyte, immune cells, etc.). Furthermore, genetic characteristic of the virus (genotypes, subgenotypes, and quasispecies) can be related to different disease outcomes, treatment options, and viral susceptibilities.

In order to prevent HCC development in chronic hepatitis patients, antiviral therapy is a treatment choice to suppress viral replication and improve general status of the patients. However, as reviewed by Papatheodoridis *et al.*^[210] current nucleos(t)ide analogs against HBV can reduce but not eliminate the risk of HCC. It is of importance to increase awareness among health-care personnel and the public in the urgency to protect new generations, particularly in endemic areas, as well as to raise the population-wide immunity by neonatal immunization program and booster and/or catch-up vaccination.

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P- Reviewer: Tashiro F, Tomizawa M **S- Editor:** Qi Y **L- Editor:** A
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