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TOPIC HIGHLIGHT

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Role of hepatitis B virus DNA integration in human hepatocarcinogenesis

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Abstract

Liver cancer ranks sixth in cancer incidence, and is the third leading cause of cancer-related deaths worldwide. Hepatocellular carcinoma (HCC) is the most common type of liver cancer, which arises from hepatocytes and accounts for approximately 70%-85% of cases. Hepatitis B virus (HBV) frequently causes liver inflammation, hepatic damage and subsequent cirrhosis. Integrated viral DNA is found in 85%-90% of HBV-related HCCs. Its presence in tumors from non-cirrhotic livers of children or young adults further supports the role of viral DNA integration in hepatocarcinogenesis. Integration of subgenomic HBV DNA fragments into different locations within the host DNA is a significant feature of chronic HBV infection. Integration has two potential consequences: (1) the host genome becomes altered ("*cis*" effect); and (2) the HBV genome becomes altered ("*trans*" effect). The *cis* effect includes insertional mutagenesis, which can potentially disrupt host gene function or alter host gene regulation. Tumor progression is frequently associated with rearrangement and partial gain or loss of both viral and host sequences. However, the role of integrated HBV DNA in hepatocarcinogenesis remains controversial. Modern technology has provided a new paradigm to further our understanding of disease mechanisms. This review summarizes the role of HBV DNA integration in human carcinogenesis.

Key words: Hepatitis B virus; Integration; Hepatocarcinogenesis; *Cis* effect; *Trans* effect; Whole genome sequencing

Core tip: A high viral load is associated with an elevated risk of hepatocellular carcinoma (HCC), and the risk remains increased in hepatitis B surface antigen-negative hepatitis B virus (HBV) and occult infections. The ability of HBV to integrate into the infected host's hepatocyte genome is one of the most important direct pro-oncogenic properties. The recent development of efficient tools for genome-wide analysis of gene expression and genetic defects has allowed a comprehensive overview of the changes occurring with HCC. Specific HBV features, including the integration of viral DNA into host chromosomes, may trigger increased genetic instability.

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INTRODUCTION

Approximately two billion people worldwide have been infected with hepatitis B virus (HBV). With more than 350 million chronic HBV carriers, this virus is one of the most common human pathogens and is a significant public health issue[1].

Liver cancer is the sixth most common cancer, and the third leading cause of cancer-related deaths[2,3]. Hepa-

tocellular carcinoma (HCC) is the most common type of liver cancer, accounting for approximately 70%-85% of cases[4]. In recent studies conducted in Asia and Northern America, the estimated risk of developing HCC was observed to increase by 25-37-fold in hepatitis B surface antigen (HBsAg) carriers compared with non-infected patients[5,6]. HBV frequently causes liver inflammation, hepatic damage and subsequent cirrhosis. The development of liver cirrhosis is recognized as a major step in HCC pathogenesis because it occurs in 80%-90% of HCCs[7]. A high viral load is associated with an elevated risk of HCC[8], and the risk remains higher in HBsAg-negative HBV and occult infections[9-11]. HBV replication has unique characteristics[1]. HBV is classified as a pararetrovirus because of its similarity to retroviruses. In fact, HBV replicates through reverse transcription of pregenomic RNA that is an intermediate replicative molecule[12]. The ability of HBV to integrate into the infected host's hepatocyte genome is one of the most important aspects of its direct pro-oncogenic properties[13-15]. Unlike retroviruses, genomic integration has no role in HBV replication and does not produce integrase enzymatic activity protein, meaning that the integrative process is likely mediated by cellular topoisomerase I activity[16].

Integrated viral DNA is found in 85%-90% of HBV-related HCCs and its presence in tumors from non-cirrhotic livers of children or young adults further supports the role of viral DNA integration in hepatocarcinogenesis[17,18]. A significant feature of chronic HBV infection is that HBV DNA fragments are integrated into different locations within the host DNA[19-23]. Tumor progression is often associated with rearrangement and partial gain or loss of both viral and cellular sequences[24-26]. Various small-scale isolated studies have suggested that HBV integration into the host genome is a random event[25]; however, integration has been observed at chromosomal fragile sites, scaffold/matrix attachment regions, and repeat/satellite sequence-rich regions[19]. Therefore, the role of integrated HBV DNA in hepatocarcinogenesis remains controversial. This review summarizes the role of HBV DNA integration in human carcinogenesis.

HCC MECHANISMS

The are three major molecular mechanisms of hepatocarcinogenesis caused by HBV infection[27]. First, the expression of viral proteins, particularly hepatitis B virus X protein (HBx), promotes cell proliferation and viability. Second, the integration of HBV DNA into the host genome alters the expression and function of endogenous genes and induces chromosomal instability. Finally, genetic damage accumulates as a result of inflammation and ongoing hepatocyte division to replace cells killed by virus-specific T cells.

Genetic alteration plays a crucial role in cancer initiation and progression. The recent development of efficient tools for genome-wide analysis of gene expression and genetic defects has allowed a comprehensive overview of the changes occurring with HCC[28,29]. Specific HBV features, including HBV DNA integration into host genome, may trigger increased genetic instability.

Table 1 Main integration sites in human genome and in hepatitis B virus DNA

Integration sites in host genome	HBV DNA
hTERT	3′ end of HBx
MLL	Pre-S2/S
RAR-b	
CCNE1	
Cyclin A2	
FN1	
ROCK1	
SENP5	
ANGPT1	
PDGF receptor	
Calcium signaling-related genes	
Ribosomal protein genes	
Epidermal growth factor receptor	
Mevalonate kinase	
Carboxypeptidase	
Platelet growth factor receptor	

HBV: Hepatitis B virus; HBx: Hepatitis B virus X protein.

ROLE OF HBV DNA INTEGRATION IN HUMAN HEPATOCARCINOGENESIS

The association between HBV DNA integration into the host genome and HCCs was first reported in the early 1980s[13,23,30]. Subsequently, many studies were performed to further investigate this association (Table 1).

The integration of HBV DNA into host cellular DNA during HBV chronic infection disrupts or promotes cellular gene expression that is important for cellular growth and differentiation. Furthermore, the expression of HBV proteins may have a direct effect on cellular functions, and may promote malignant transformation. Integration events are thought to precede tumor development because they are found in chronic hepatitis patients and during the acute infection stage[31].

Technological limitations of PCR and Southern blot-based methods restricted previous studies that attempted to characterize the most common HBV integrant(s) in a small number of patients[15,32]. HBV has a large number of mutations at both the nucleotide and structural levels, and the lack of prior knowledge of HBV sequences in each sample may lead to PCR failure and false-negative results. This occurs when the primers are designed for deleted or polymorphic sites on the HBV genome. Recently, two studies reported "short-read" whole genome DNA paired-end sequencing of four and eighty-eight HCC patients[33,34]. Integration sites could only be inferred from paired-end reads containing both human and viral sequences, because of the limitations of the short reads generated using these platforms. Indirect roles have been proposed because the lack of identification of a dominant oncogene encoded by HBV, including insertional

activation of cancer-related genes from HBV integration, induction of genetic instability by viral integration or HBx, and long-term effects of viral proteins that enhance immune-mediated liver disease.

Integration has two potential consequences: (1) the host genome becomes altered ("*cis*" effect); and (2) the HBV genome becomes altered ("*trans*" effect). The cis effect includes insertional mutagenesis, which can potentially disrupt host gene function or alter host gene regulation [*e.g.*, telomerase reverse transcriptase (TERT)][35]. Despite drastic rearrangements, the coding regions of PreS2 and HBx were generally conserved and could be transcribed[36]. Hence, these two HBV proteins may have a *trans* role in hepatocarcinogenesis[37-39].

CIS EFFECT

The main integration sites in the human genome and the preferred integrating region within the HBV genome have been researched extensively.

HBV DNA integration occurs randomly within human genomes, and may involve multiple sites in different chromosomes[25]. Thus, HBV behaves like an insertional, non-selective mutagenic agent. The important host genome rearrangements associated with viral integration suggest that the main oncogenic effect is from the induction of higher genomic instability[40]. Most reported integration events occur near or within fragile sites or other repetitive regions, such as the Alu sequences and microsatellites that are prone to instability, tumor development, and progression[22]. Integration of HBV DNA sequences begins in the early stages of acute infections, and multiple integrations have been detected in chronic hepatitis tissues. Clonal integrated HBV sequences have been observed in approximately 80% of HBV-related HCCs[41]. Viral insertion sites have been mapped in multiple regions on virtually all chromosomes, suggesting a random distribution throughout the host genome. HBV insertions are commonly associated with large genetic alterations that may lead to the abrogation of control mechanisms that safeguard chromosomal integrity[42-45]. Similar to retroviral proviruses, HBV DNA targets actively transcribed chromosomal regions within genes or in the immediate vicinity. Sequence analysis of multiple viral-host junctions have identified cellular coding regions within several kbps in 90% of cases, with frequent targeting of gene families involved in cell survival, proliferation and immortalization including: hTERT, the PDGF receptor, MLL, calcium signaling-related genes and ribosomal protein genes[15]. These findings favor the view that viral insertion induces the first genetic alteration in tumor development. Target genes may play a role in hepatocarcinogenesis, which was previously shown for HBV insertions into the retinoic acid receptor b (RAR-b) and the cyclin A2 genes[46,47].

Among the numerous viral integration sites described, some notable regions include the tyrosine-protein-kinase domain of the epidermal growth factor receptor gene[48], the mevalonate kinase gene[49,50], the carboxypeptidase gene[51], platelet growth factor receptor genes[15] and *hTERT*.

The HBx gene in the HBV genome tends to be the most common region, but the most common integration sites in the human genome are not fully identified. Several integration sites in the human genome such as *TERT*, *MLL4*, *CCNE1*, *FN1*, *ROCK1* and *SENP5* have been reported[33-52]. *TERT* encodes a telomerase reverse transcriptase, which plays an essential role in overriding cellular senescence. Its dysregulation in somatic cells is linked to carcinogenesis[53]. *MLL4* encodes a histone methyltransferase that plays a critical role in gene expression and epigenetics in cancer cells. The translocation breakpoint of the intron 3 region of *MLL4* is one of the preferential targets for HBV DNA integration and may be involved in liver oncogenesis[54]. *CCNE1* encodes cyclin E1, which is required for cell cycle G1/S transition. *FN1* encodes fibronectin, a component of the extracellular matrix that is involved in cell adhesion and migration processes. The protein encoded by *ROCK1* can activate LIM kinase, and inhibits actin-depolymerizing activity by phosphorylating cofilin. *SENP5* encodes a protease specific for SUMO proteins, and is required for numerous biological processes. All of these genes are upregulated in malignant tissues[34]. Hence, HBV integration into these genes may cause HCC.

Whole genome sequencing (WGS) of a large cohort has provided an opportunity to identify novel recurrent integrations. In addition to the confirmation of recurrent HBV integration into the *MLL4* (n = 9) and *TERT* (n = 18) loci accompanied by upregulation of gene expression, recurrent integration events were observed at the *CCNE1* (n = 4), *SENP5* (n = 3), and *ROCK1* (n = 2) loci[34]. *CCNE1* expression was, on average, 30-fold higher in tumors with HBV integration compared to the normal controls. Cyclins are mainly involved in regulating the cell cycle in eukaryotic cells, and are major targets for oncogenic signals. HBV integration at the *CCNE1* locus has provided at least one molecular mechanism driving aberrant cell cycle control leading to HCC. Currently, three genome-sequencing studies have been published that analyzed HBV integration events. Genome sequencing of four HCC patients identified 255 HBV integration sites in the three HBV-positive patients including the *MLL4* locus in one sample and the *ANGPT1* locus in another[33]. RNA sequencing revealed a distinct transcriptional impact of viral integration. HBV DNA integration into the third exon of *MLL4* resulted in a human-viral fusion transcript, and a 20-fold increase in *MLL4* transcription in comparison to the adjacent normal liver tissue. For the *ANGPT1* gene, HBV DNA was inserted into 10-kb upstream of the promoter region, leading to a greater than eightfold elevation in *ANGPT1* expression. In a genome sequencing study of 27 HCCs, including 11 HBV-associated HCC, 14 HCV-associated HCC, and two cases that were unrelated to viral infection, the average proportion of the *TERT* integration sites (41%) was higher than that of other integration sites. These findings are consistent with previous reports of recurrent HBV integration at the *TERT* locus[55].

Preferential HBV integration into gene promoters ($P < 0.001$), and significant enrichment of integration into chromosome 10 ($P < 0.01$) was observed in the tumors. Integration into chromosome 10 was significantly associated with poorly differentiated tumors ($P < 0.05$). In particular, recurrent integration into the *TERT* promoter was correlated with increased TERT expression[56].

We found that HBV DNA integration enhanced host chromosomal instability leading to large inverted duplications, deletions and chromosomal translocations[32]. Many of these chromosomal segments contain genes encoding key factors in liver carcinogenesis, such as p53, Rb, Wnt/b-catenin, cyclins A and D1, TGFb, and Ras[57].

TRANS EFFECT

Integrated viral sequences may contribute "*in trans*" to tumorigenesis through the production of truncated and mutated HBx or preS2/S proteins, though they cause defective replication. These proteins may impact HCC development by disrupting cellular gene expression control or by activating oncogenic signaling pathways.

The HBx protein is a multifunctional regulator of viral and cellular genes that interferes with viral replication and proliferation. HBx and Pre-S2/S regulatory proteins that are generated from integrated viral sequences are involved in hepatocyte transformation. Moreover, HBx and truncated Pre-S2/S have been shown to be effective transactivators of cellular and viral genes and are involved in signal transduction pathways, cell cycle control and transcriptional regulation[56,58].

The C-terminal region of HBx, produced by HBx truncation, contributes to HCC development. It has been suggested that the C-terminal region is required for reactive oxygen species (ROS) production and 8-oxoguanine (8-oxoG) formation, which are biomarkers of oxidative stress. Oxidative stress and mitochondrial DNA damage play an important role in the development of HCC[59]. Other studies have found that HBx C-terminal truncation, particularly involving 24 amino acids, plays a role in enhancing cell invasiveness and metastasis in HCC by activating MMP10 through C-Jun signaling[60]. Also, HBx C-terminal truncation was closely related to the overexpression of centromere protein A in HCC[61]. In addition, HBx C-terminal truncation directly regulates miRNA transcription and promotes hepatocellular proliferation[62].

Most HBV-related HCCs have integrated viral genomic sequences, including the HBx gene. Although the integrated forms of HBx are frequently rearranged and show numerous point mutations, deletions or truncation, integrated HBx may encode functionally active proteins with transactivating ability[31,41]. Characterization of HBx expression in malignant hepatocytes and infected liver tissues has been often hampered by the difficulty in obtaining valid high-affinity anti-HBX antibodies for immunodetection[63]. Despite this, the expression of HBx is maintained through multistage hepatocarcinogenesis from pre-neoplastic nodules or foci of transformed hepatocytes to HCC[64,65].

Evidence of transcriptional activity at integrated X sequences has been demonstrated in tumors and chronically infected livers[66,67] and may be correlated with the detection of the X protein in human HCCs[68]. It was suggested that downstream cellular sequences contribute to activated expression and/or enhanced transactivating capacities of the integrated HBV sequences[58,69]. The X gene product transactivates homologous and heterologous transcriptional enhancers and promoter sequences. In the meantime, expression of cellular genes is activated *"in trans"* from increased X gene products. Many clones preserved transactivation activity in spite of the truncation at the 3' end of the X ORF[67]. The cDNA structure of X mRNA from integrated HBV DNA suggested X-cell fusion mRNA.

The preferred region within the HBV genome involved in integration and viral structural alteration is located at nucleotides 1600-1900 around the 3'-end of HBx and the 5'-end of the Precore/Core genes, where viral replication and transcription is initiated. Upon integration, the 3'-end of HBx is frequently deleted and HBx-human chimeric transcripts, which can be expressed as chimeric proteins, are commonly observed[56]. The 3'-end of the HBx gene is the preferred region for human genome integration[34,52,70], leading to the C-terminal truncated form of HBx, and is an important mechanism in HBV-related hepatocarcinogenesis.

Recently, WGS was performed on a large cohort of HCC patients with 81 HBV-positive, seven HBV-negative HCC samples and adjacent normal tissues to survey HBV integration in liver cancer genomes[34]. A systematic and in-depth bioinformatics analysis was performed to study HBV integration. The 399 detected HBV integration events occurred more frequently in tumors (344 events) than the normal controls (55 events), and represented a 6.3-fold increase. The HBV genome break points were also examined, and 40% of the break points were restricted to an 1800-bp region of the HBV genome where the viral enhancer, the X gene and the core gene are located. This viral breakpoint may facilitate the formation of human-viral fusion proteins and create cis-regulatory effects on expression of downstream genes that disturb the host gene regulatory network.

Some HCC patients do not have detectable hepatitis B surface antigen in their serum, but have low levels of serum HBV DNA and fragments of HBV DNA in their genomic cellular DNA (occult HBV infections). The prevalence and molecular status of occult HBV in HCC patients has been investigated in many studies in patients from different regions worldwide[10,71,72]. In HBsAg-negative HCC patients, HBV DNA was detected in neoplastic and/or adjacent non-neoplastic liver tissue in almost half of patients, some of which were anti-HCV positive[73]. In some patients, positivity for anti-HBc antibodies was the only marker of HBV infection. Covalently closed circular HBV DNA may be detected in the liver of some patients, indicating persistence of the viral genome template for transcription and replication. An observational cohort study showed that HCC develops more commonly in oc-

cult HBV patients among HBsAg-negative patients with chronic hepatitis C.

In addition to genetic and genomic perturbations, HBV integration is also associated with various clinical parameters including disease occurrence at younger age, higher levels of AFP and poor overall survival[54]. This suggests an association between viral DNA integration and a more aggressive pathogenesis of HCC.

Beside genomic alterations, epigenetic factors, such as methylation-associated gene silencing, have been shown to be involved in the deregulation of cellular function in HCC. The HBV genome is almost completely unmethylated in the early stages of carcinogenesis, from chronic active hepatitis to hepatic cirrhosis, while it becomes more methylated in the established liver tumors, both in patients and in cultured cancer cell lines[74].

CONCLUSION

The multistep development of liver cancer is associated with the accumulation of genetic and epigenetic changes. The long latency of HCC development following primary HBV infection reflects an indirect oncogenic pathway. Evidence of multiple cooperative mechanisms during neoplastic transformation is increasing. Genetic instability, which is particularly high in HBV-related HCCs, may be related to HBV integration.

The integration of HBV has the primary *cis* effect of altering gene regulation. Sequence variations and structural alterations of the HBV genome that modify viral protein structure, function and integration events generate novel HBx-human chimeric proteins that may exert a *trans* effect by facilitating host immune surveillance evasion and/or that contribute to tumorigenesis.

Next generation sequencing technology has provided a new paradigm for understanding disease mechanisms. WGS and whole exome sequencing efforts have led to the discovery of previously unknown somatic variations in HCC, such as point mutations in chromatin remodeling genes and recurrent HBV integrations. A large number of data sets from genome wide association studies may need further investigation. Additional research into the development and treatment of resistant HBV strains is warranted.

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