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Somatic Mutations, Viral Integration and Epigenetic Modification in the Evolution of Hepatitis B Virus-Induced Hepatocellular Carcinoma

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Abstract: Liver cancer in men is the second leading cause of cancer death and hepatocellular carcinoma (HCC) accounts for 70%-85% of the total liver cancer worldwide. Chronic infection with hepatitis B virus (HBV) is the major cause of HCC. Chronic, intermittently active inflammation provides "fertile field" for "mutation, selection, and adaptation" of HBV and the infected hepatocytes, a long-term evolutionary process during HBV-induced carcinogenesis. HBV mutations, which are positively selected by insufficient immunity, can promote and predict the occurrence of HCC. Recently, advanced sequencing technologies including whole genome sequencing, exome sequencing, and RNA sequencing provide opportunities to better understand the insight of how somatic mutations, structure variations, HBV integrations, and epigenetic modifications contribute to HCC development. Genomic variations of HCC caused by various etiological factors may be different, but the common driver mutations are important to elucidate the HCC evolutionary process. Genome-wide analyses of HBV integrations are helpful in clarifying the targeted genes of HBV in carcinogenesis and disease progression. RNA sequencing can identify key molecules whose expressions are epigenetically modified during HCC evolution. In this review, we summarized the current findings of next generation sequencings for HBV-HCC and proposed a theory framework of *Cancer Evolution and Development* based on the current knowledge of HBV-induced HCC to characterize and interpret evolutionary mechanisms of HCC and possible other cancers. Understanding the key viral and genomic variations involved in HCC evolution is essential for generating effective diagnostic, prognostic, and predictive biomarkers as well as therapeutic targets for the interventions of HBV-HCC.

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INTRODUCTION

Liver cancer in males is the fifth most frequently diagnosed cancer and the second most frequent cause of cancer-related death worldwide; in females, it is the seventh most commonly diagnosed cancer and the sixth leading cause of cancer-related death. Hepatocellular carcinoma (HCC) represents the major histotype, accounting for 70% to 85% of the total liver cancer burden worldwide [1]. Annually, over 600,000 new HCC cases are diagnosed worldwide [2]. Chronic infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) is the major cause of this malignancy. Chronic HBV infection accounts for about 60% of the total liver cancer in developing countries and for about 23% in developed countries [3]. Other factors such as alcohol consumption, smoking, aflatoxin B exposure, diabetes, obesity, and non-alcoholic fatty liver disease may be also associated with an increased risk of HCC [4]. Somatic mutations, such as p53 mutation, HBV integration to the fragile sites of host genome, and epigenetic silencing of tumor suppressor genes are possible molecular mechanisms underlining hepatocarcinogenesis [5]. Deregulation of non-coding RNAs is also involved in HCC development [6, 7]. Genomic variations of HCC caused by various etiological factors might be different, but the common genomic variations should be important to elucidate the HCC evolutionary process. Understanding genetic and epigenetic etiological factors of HBV-induced HCC (HBV-HCC) may pave the way for the development of efficient prophylactic and therapeutic interventions to reduce HCC burden.

To elucidate the role of molecules and genetic alterations in hepatocarcinogenesis, research methods and techniques are important. The traditional biological methods such as PCR and southern blot [8, 9] are constantly used to identify genetic predisposition, mutations, and HBV integrations, which present a lot to understand the association of certain genes or their modifications with cancers. However, these methods are less cost-effective. Neither traditional PCR-based methods nor microarray is technically sufficient to identify and quantify key molecular events such as HBV integration sites in a genome-wide scale. In the recent decade, with the emergence of advanced high throughput platforms such as next-generation sequencing (NGS) technol-

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ogy, cancer research has stepped into a new era. NGS, also named deep sequencing, high throughput sequencing, brings striking impacts for genetic and biological research by the unbiased and comprehensive sequencing capacity. Just as Roukos mentioned, the era of NGS has provided a comprehensive and integral view of cancer genome architecture [10]. High throughput and sharp decrease in reagent volume needed for each sample, which greatly reduces the cost per nucleotide, are the advantage of NGS. NGS including whole genome sequencing (WGS), whole exome sequencing (WES), and RNA sequencing can be used to sequence human genomes and transcriptomes, thus establishing a mutation catalog for many diseases. These new technologies have enabled us to screen for cancer-related molecules or events in a more comprehensive and unbiased way, providing a higher success rate to identify key mutations involved in the crucial evolutionary process of carcinogenesis. In the current review, we updated somatic mutations of HCC caused by various etiological factors, the integrations of HBV into host genome, the deregulation of non-coding RNAs, and epigenetic modifications identified by NGS to interpret the molecular mechanisms of HCC development. Based on the current knowledge of HBV-induced HCC, we propose a theory framework of *Cancer Evolution and Development* or termed as "*Evo-Dev of Cancer*" to characterize evolutionary regulation of cancers.

HBV MUTATIONS AND HCC EVOLUTION

HBV genotype/subgenotype and insufficient antiviral immunity contribute to the establishment of chronic HBV infection in adults and early children, respectively [11, 12]. Human liver is a rich source of innate and adaptive immune cells, which constitute the framework of immunity combined with cytokines/chemokines. Tumor microenvironment is a dynamic system, which is largely orchestrated by inflammatory molecules, stromal cells, immune cells, and extracellular matrix. The imbalance of inflammatory molecules in the inflammatory microenvironment may promote the progression of HBV-related diseases [13]. Chronic inflammation caused by insufficient immunity to HBV antigens is indispensible for the occurrence of HCC. Human leukocyte antigens (HLAs) are essential for immune recognition and response, while their genetic predispositions may contribute to immune imbalance upon HBV infection, leading to chronic HBV infection, immune selection of HCC-risk HBV mutations, and subsequent HCC [14]. HBV evolution promoted by chronic inflammation can be considered as a process of "mutation-selection-adaptation" during hepatocarcinogenesis. Cytokines storm induced by chronic inflammation or antiviral cytokines such as interferon can up-regulate the expression of activation-induced cytidine deaminase (AID) and apolipoprotein B mRNA editing enzyme, catalytic polypeptides (APOBECs), while these enzymes can specifically catalyze the irreversible cytidine and deoxycytidine deamination to convert bases from cytosine (C) to uracil (U) on RNA and DNA, respectively [15]. HBV DNA can be easily degraded by the endogenous enzymes when viral DNA forms single stranded DNA (ssDNA) during viral replication [16-20]. Viral mutations facilitate immune escape of HBV in immunocompromised microenvironment and promote hepatocarcinogenesis. The HCC-related HBV mutations may up-regulate HBV expression and increase its virulence. It has been shown that HBV mutations in the S genes, especially a substitution mutation of glycine to arginine at site 145 (G145R), are associated with immune escape [21, 22]. The HBV A1762T/G1764A-based mutations have been suggested to be independently associated with HCC development, and effectively predict poor prognosis of HCC [15, 23, 24]. HBx mutants with A1762T/G1764A, T1753A, and T1768A in primary human hepatocytes and HCC cells significantly up-regulate S-phase kinase-associated protein 2 (SKP2), and then down-regulate p21, which have been demonstrated to be associated with cell cycle progression and proliferation [25]; the full-length HBV genome with the corresponding mutations is also able to enhance SKP2 transcription by activating the E2F1 transcription factor in primary human hepatocytes and HCC cells, down-regulate cell cycle inhibitors, and accelerate cellular proliferation [26]. Thus, HBV mutation should be an important cause of HBV-HCC. These HCC-related HBV mutations are positively selected by incomplete immunity in the microenvironment and in turn promote the growth and aggressiveness of HCC. Thus, we believe that HBV mutations are generated during the evolutionary process of hepatocarcinogenesis, and APOBECs play important roles in the evolution of HBV genome *via* promoting the HBV mutations. AID, APOBECs, and their unrevealed analogues not only promote the mutations of HBV genome, but also promote somatic mutations of key genes in human genome.

HCC-RELATED SOMATIC MUTATIONS IDENTIFIED BY DEEP SEQUENCING

The advent and rapid development of NGS enabled more convenient and accurate discoveries of cancer-related somatic mutations in an unbiased and comprehensive way. Genomic deep sequencing of blood samples from hematological cancers, such as acute myeloid leukemia (AML) [27, 28], has been proven to be a big success. However, the applications of this technology are questioned for solid tumors because solid tumor tissues contain "normal" cells such as fibroblasts, blood vessels, and immune cells. Normal DNAs in tumor tissues may introduce biases in DNA genomic analysis, leading to inaccurate identification of genetic information in tumor cells [29]. Despite these shortcomings of NGS studies in solid tumors like HCC, many genes and related signaling pathways involved in hepatocarcinogenesis have been identified by this method. Important HCC-related somatic mutations are found in critical genes such as RNA editing genes (*ADAR1, ADAR2, KHDRBS2,* and *RTL1*) [30-33], chromatin remodeling genes (*ARID1A, ARID1B,* and *ARID2*) [34-38], DNA binding genes (*HOXA1)* [36], growth factor signaling pathway genes (*CDH8, CDK14, CNTN2, ERRFI1, RPS6KA3, P62,* and *PROKR2*) [34-36, 39, 40], transcriptional regulation genes (*AXIN1, CCNG1, CTNNB1, IRF2, NFE2L2, PARP4, PAX5, ST18, TP53, TRRAP,* and *ZNF717*) [30, 35-39, 41-44], cell structure modification genes (*FLNA* and *VCAM1*) [36, 39], and epigenetic modification genes (*MLL3*) [34, 41], JAK/STAT pathway genes (*JAK1* and *JAK2*) [30, 41], and other unknown functional genes. (Table **1**) summarizes

the characteristics of major mutated genes (mutation frequencies of >3%) that have been found to be associated with HCC by NGS platforms.

However, several important scientific issues concerning cancer-related somatic mutations remain to be addressed: first, as cancer-related somatic mutations are revealed *via* comparing to the adjacent, pathologically normal non-cancerous tissues, it remains unknown if these identified somatic mutations are the causes or consequences of hepatocarcinogenesis. Somatic mutations in cancers can be generally classified as driver and passenger mutations. Driver mutations are positively selected during cancer evolution and indispensible for cancer development. Most somatic mutations are passenger mutations that are not subject to positive selection and are not directly associated with carcinogenesis. A series of functional experiments are needed to identify the nature of a given somatic mutation. Second, the mutations usually appear inconsistently in coding or non-coding regions of a given gene, furthermore, the frequencies of some HCC-related somatic mutations may vary greatly in HBV-HCC tissues, ranging from 1.8%-5.8% of *ARID2* to 71.7% of *ADAR1* [31, 34, 36]. The unstable frequencies of a given somatic mutation and generally low frequencies of cancer-related somatic mutations make them unsuitable to serve as direct biomarkers for the diagnosis and prognosis prediction of HBV-HCC. Future of their application in clinical settings should be the targeted therapy for HBV-HCC, as the HCC-related somatic mutations clearly affect some important signaling pathways, such as Wnt/β-catenin signaling and ras/mitogen-activated protein kinase (MAPK) pathways [35]. Some of these signaling pathways are indispensible for HCC progression. The functional interpretations of these genes can be found *via* the 'GO' (Gene Ontology) annotations (http://genecards.org/). It remains to be clarified if HBV-HCC with specific somatic mutations is quite suitable for a targeted therapy to retard HCC progression *via* blocking a given pathway or more.

HBV INTEGRATION REVEALED BY DEEP SEQUENCING

Chronic infections with HBV and/or HCV are the well established causes of HCC [45]. HCV, a positive-sense single-stranded RNA virus, cannot integrate to human genomes. No viral-human genomic integration has been detected in HCV-related HCC so far. HBV is a DNA virus with a relaxed circular, partially double-stranded genome, which contains 4 overlapping open reading frames (ORFs) encoding surface (S), core (C), polymerase (P) and X proteins. It was firstly proposed in 1981 that HBV integration into the human genome occurred in the infected hepatocytes since early stage of HBV infection [46]. HBV integration is considered as an important mechanism for virus-induced hepatocarcinogenesis, especially in younger HCC patients without cirrhosis [47]. Some studies also indicate that the number of HBV integrations is significantly associated with patient survival [48]. In HBV-HCC patients, 85% - 90% integration events can be found in liver cancer tissues [49]. Therefore, it is reasonable to hypothesize that HBV integrations play important roles in the process of hepatocarcinogenesis.

Location of Cleave Sites of HBV Integrations in Human Genome

Previous studies have shown that HBV integration can be detected from tumor and adjacent non-tumor tissues of HBV-positive HCC patients, but not HBV-negative samples. However, the HBV integrations in cancer tissues or adjacent tissues are different. A study has shown that HBV integration is more frequent in tumors (86.4%) than in adjacent liver tissues (30.7%) [48]. On the contrary, another study claimed that the occurrence of HBV integration in adjacent tissues (254/296, 85.8%) was more often than those in tumor tissues (42/296, about 14.2%) [50]. The cause of this discrepancy is unknown. Preferable clonal expansion of some hepatocytes in a suitable microenvironment may explain this disparity of HBV integration in normal and HCC tissues [51].

It is traditionally believed that HBV randomly integrates into the human genome [47, 49]. However, this standpoint has been recently challenged by large-scale analyses of HBV insertion sites, such as Alu-PCR or NGS. According to recent findings, HBV integration prefers to occur in actively transcribed chromosomal regions and the repetitive sequences, including long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), and Alu sequences [44, 48, 49, 52]. Recently, high-throughput technologies using different platforms have discovered that HBV integration favors chromosome 10 [52] and chromosome 17 [50]. Alternations in chromosomal stability and changes in copy numbers are considered to be the characteristics of HBV integration events in HCC. For example, copy number loss is present at a locus adjacent to the cluster of caspase genes on chromosome 11 [44], whereas copy number variations are present in HBV integration breakpoints [48]. The variations of copy number located within or near the integration sites suggest that HBV integration may induce chromosomal instability, one of the major mechanisms of hepatocarcinogenesis. Another possible mechanism of HCC development is that some oncogenes or tumor suppressor genes may be affected by chimeric HBV-human fusion genes [48]. The characteristics of HBV integration in HCC identified by deep sequencing are listed in (Table **2**).

Genes and Pathways Associated with HBV Integration Identified by Deep Sequencing

The first report using PCR to study HBV integration in human HCCs has demonstrated that a gene encoding telomerase reverse transcriptase (*TERT*) located at chromosome 5p15.33 is a HBV integration breakpoint [46]. Since then, more and more targeted genes have been detected by PCR. *TERT* and myeloid/lymphoid or mixed-lineage leukemia 4 (*MLL4*) are the most preferential targets of HBV integration. *TERT* plays an important role in overriding cellular senescence, which is often up-regulated in most cancer cells [53]. *MLL4* encodes a histone methyltransferase, which has critical roles in epigenetic modification of cancer genome [54]. As traditional methods are not sufficient to identify and quantify HBV integration sites in a comprehensive scale, NGS has been used to screen for HBV integrated sites/genes in HCC patients. Recently, a WGS study has confirmed *TERT* gene as a breakpoint of HBV integration. It has also identified one

Table 1. Genes associated with hepatocellular carcinoma identified by next generation sequencing.

Gene	'GO' Annotations	Location	Technology	Etiology of HCC	Ref.
ADAR1	Double-stranded RNA binding and double- stranded RNA adenosine deaminase activity	1q21.3	RNA-Seq	Unknown	[31, 32]
ADAR2	Double-stranded RNA binding and double- stranded RNA adenosine deaminase activity	1q21.3	RNA-Seq	Unknown	[31]
ADCY2	Adenylate cyclase activity and protein heterodimerization activity	5p15.31	WGS	HBV	[41]
AMPH	Protein binding andprotein domain specific binding	7p14.1	WES	HBV	[36]
ARID1A	Transcription coactivator activity and ligand-dependent nuclear receptor binding	1p36.11	WGS, WES, RNA-Seq	HBV, HCV, alcohol, other causes	[34-36]
ARID1B	Transcription coactivator activity and DNA binding	6q25.3	WGS	HBV, HCV, other causes	[34]
ARID2	DNA binding and zinc ion binding	12q12	WGS, WES	HBV, HCV, other causes	[34, 37]
ATM	Protein serine/threonine kinase activity and protein complex binding	11q22.3	WGS	HBV, HCV, other causes	[34]
AXIN1	Protein kinase binding and protein homodimerization activity	16p13.3	WGS, WES	HCV	[30, 35, 41]
C18orf34	Unknown	unknown	WES	HBV	[36]
CACNA2D4	Voltage-gated ion channel activity and calcium channel activity	12p13.33	WGS	HBV	[41]
CCNG1	Protein binding and protein domain specific binding	5q34	WGS, WES	HBV	[40]
CDH8	Calcium ion binding	16q21	WGS, WES	alcohol	[35]
CDK14	Cyclin binding and cyclin-dependent protein serine/threonine kinase activity	7q21.13	WES	HBV	[36]
CNTN2	Glycoprotein binding and identical protein binding	1q32.1	WGS, WES	HBV	[39]
COL11A1	Extracellular matrix binding	1p21.1	WGS	HBV	[41]
CSMD3	Unknown	8q23.3	WES	HBV	[36]
CTNNB1	Protein kinase binding and sequence-specific DNA binding transcription factor activity	3p22.1	WGS, WES	HBV	[35, 37, 38, 41]
DMXL1	Unknown	5q23.1	WES	HBV	[37, 38]
DSE	Chondroitin-glucuronate 5-epimerase activity	6q22.1	WES	HBV	[36]
ELL	Protein binding	19p13.11	WES	HBV	[36]
ELMO1	Phospholipid binding and SH3 domain binding	7p14.1	WES	HBV	[36]
EPS15	SH3 domain binding and calciumion binding	1p32.3	WGS	HBV	[41]
ERRFI1	Rho gtpase activator activity and protein kinase binding	1p36.23	WGS	HBV, HCV, other causes	[34]
FAM5C	Unknown	unknown	WGS	HBV	[41]
FLNA	Protein homodimerization activity and signal transducer activity	Xq28	WGS, WES	HBV	[39]
HOXA1	Sequence-specific DNA binding and sequence-specific DNA binding transcription factor activity	7p15.2	WES	HBV	[36]
IGSF10	Unknown	3q25.1	WGS	HBV, HCV, other causes	[34]
IRF2	Regulatory region DNA binding and sequence-specific DNA binding transcription factor activity	4q35.1	WES	alcohol	[35]

(Table 1) contd….

Gene	'GO' Annotations	Location	Technology	Etiology of HCC	Ref.
JAK1	Growth hormone receptor binding and protein tyrosine kinase activity	1p31.3	WGS	HBV	[41]
JAK2	Protein kinase activity and receptor binding	9p24.1	WGS, WES	HCV	[30]
KHDRBS2	SH2 domain binding and protein heterodimerization activity	6q11.1	WGS, WES	HCV	[30]
LRP1B	Low-density lipoprotein receptor activity and calcium ion binding	2q22.2	WGS	HBV	[41]
MLL3	Unknown	7q36.1	WGS	HBV, HCV, other causes	[34, 41]
NEK8	Protein serine/threonine kinase activity and metal ion binding	17q11.2	WGS, WES	HCV	[30]
NFE2L2	Sequence-specific DNA binding transcription factor activity and protein domain specific binding	2q31.2	WES	alcohol	[35]
NLRP1	Cysteine-type endopeptidase activator activity involved in apoptotic process and enzyme binding	17p13.2	WES	HBV	[37, 38]
P62(DCTN4)	Protein N-terminus binding	5q33.1	WGS, WES	HBV	[40]
PARP4	NAD+ADP-ribosyltransferase activity and enzyme binding	13q12.12	WGS, WES	HBV	[39]
PAX5	DNA binding and sequence-specific DNA binding transcription factor activity	9p13.2	BGS	Unknown	[42]
PROKR2	Neuropeptide Y receptor activity	20p12.3	WGS, WES	alcohol	[35]
RPS6KA3	Protein serine/threonine kinase activity and protein kinase activity	Xp22.12	WES	alcohol	[35]
RTL1	Unknown	14q32.2	WES, RNA-Seq	SB induced HCC	[33]
SAMD9L	Unknown	7q21.2	WES	HBV	[36]
SLC10A1	Bile acid:sodium symporter activity	14q24.2	WGS	HBV	[41]
SPAG17	Unknown	1p12	WES	HBV	[36]
ST18	DNA binding and sequence-specific DNA binding transcription factor activity	8q11.23	RC-seq	Unknown	[43]
TMEM2	Unknown	9q21.13	WES	HBV	[36]
TMEM35	Molecular_function	Xq22.1	WES	HBV	[36]
TP53	Identical protein binding and sequence-specific DNA binding transcription factor activity	17p13.1	WGS, WES	HBV, HCV	[35, 37, 41, 44]
TRRAP	Transcription coactivator activity and phosphotransferase activity, alcohol group as acceptor	7q22.1	WGS, WES	HCV	[30]
VCAM1	Integrin binding and cell adhesion molecule binding	1p21.2	WES	HBV	[36]
WWP1	Ubiquitin-protein ligase activity and protein binding	8q21.3	WGS	HBV, HCV, other causes	[34]
ZIC3	Sequence-specific DNA binding and sequence-Specific DNA binding transcription factor activity	Xq26.3	WGS	HBV, HCV, other causes	[34]
ZNF226	DNA binding and zinc ion binding	19q13.31	WGS	HBV, HCV, other causes	[34]
ZNF717	DNA binding and zinc ion binding	3p12.3	WGS, WES	HBV	[39]

BGS, Bisulfate genomic sequencing; 'GO', Gene Ontology (http://www.genecards.org/); HBV, hepatitis B virus; HCV, hepatitis C virus; RC-seq, retrotransposon capture sequencing; RNA-seq, RNA sequencing; SB, Sleeping Beauty; WES, whole exome sequencing; WGS, whole genome sequencing.

Table 2. Characters of HBV integrations analysis by next generation sequencing.

Major Genes	Sample Type	T/Total Integ -rated sites	Platforms	Preferred Human Integrated Chr.	Preferred Breakpoints on HBV Genome	Ref.
TERT, *FAS*, *MSMB*	T *vs*. NT (48 pairs of HBV+)	Total: 97 unknown	WGS	Chr10	3′-end of HBx region (nt.1600 – nt.1900) and 5′-end of the Precore /Core region	[52]
MLL4, *ANGPT1*	T *vs*. NT (3 of HBV+, 1 of HBV-)	148/255	WGS, RNA-Seq	Unknown	A region between nt.1500-nt.2000	[44]
TERT, *MLL4*, *CCNE1*, *SENP5*, *ROCK1*, *FN1*	T *vs.* NT (81 of HBV+, 7 of HBV-)	344/399	WGS, RNA-Seq	Unknown	Over 40% located at about nt.1800	[48]
CCNG1, P62	T *vs.* NT (1 of HBV+)	18/18	WGS, WES	Unknown	Unknown	[40]
TERT, *MLL4*, *CCNE1*	T (28 of HBV+)	246/246	HIVID, WGS	Unknown	33.5% located at nt.1500-nt.2000, 38.9% located at pre-S1 region	[55]
FN1, *PHACTR4*, *RBFOX*, *SMAD5*, *TERT*	T *vs.* NT (40 pairs of HBV+)	42/296	MAPS	Chr17	Half of chermic human-HBV DNA truncated between nt.1500-nt.2000	[50]
TERT	T *vs.* lymphocytes (11 of HBV+, 2 of HBV-)	Total: 23 unknown	WGS	Unknown	The downstream region of the HBx region	[34]

bp, base pairs; Chr., chromosome; HBV+, HBV positive; HBV-, HBV negative; HBx, HBV X protein; HIVID, high throughput viral integration detection; MAPS: massive anchored parallel sequencing; NT, non-tumor tissues; RNA-seq, RNA sequencing; T, tumor tissues; WES, whole exome sequencing; WGS, whole genome sequencing.

oncogene (*ERBB4*) and six tumor suppressor genes (*CCNA2*, *MSMB*, *MYO18B*, *AIP*, *NTN1* and *FAS*) as the integration sites [52]. In addition, *MLL4*, which was ever implicated in the *p53* tumor suppressor pathway, has also been identified as a hot HBV integration site by WGS (>80×coverage) and transcriptome sequencing [44]. However, the findings of these two studies need to be further confirmed because of their small sample sizes. The first study included 11 HBV-related HCC, 14 HCV-related HCC, and 2 HBV-, HCV-negative HCC subjects; while another one only included 4 patients (3 HBV-positive and 1 HBV-negative patients). Recently, a comprehensive study using WGS with a sample size of 81 HBV-positive and 7 HBV-negative HCC tissues has shown that HBV integration events occur repeatedly at the loci of *TERT*, *MLL4*, and *CCNE1* while viral-human integrations may alter chromosome stability and gene expression levels and tend to be associated with the prognosis of HBV-related HCC [48]. Furthermore, novel genes associated with HBV integration such as *FN1*, *SENP5*, and *ROCK1* have also been identified [48]. A massive anchored parallel sequencing (MAPS) study has shown that *ARHGEF12* [Rho guanine nucleotide exchange factor (GEF) 12], *CYP2C8* (cytochromeP450, family 2, subfamily C, polypeptide 8), *FN1*, *PHACTR4* (phosphatase and actin regulator 4), *PLXNA4* (Plexin A4), *RBFOX1* [RNA binding protein, fox-1 homolog (C. elegans) 1], *SMAD5* (*SMAD* family member 5), and *TERT* are on the list of recurrent HBV targets [50]. Notably, HBV integration at *FN1* gene has only been found in normal or adjacent tissues, but not in tumor tissues, suggesting that HBV cleaved site at *FN1* gene is not a random event [48, 50]. An improved deep sequencing technology with higher sensitivity and specificity, named high-throughput viral integration detection (HIVID) that can enrich HBV sequencing by a set of HBV probes, also suggests that HBV integrations cleave at *TERT*, *MLL4*, and *CCNE1* [55]. In addition, two novel integrated sites located in the introns of *CCNG1* and *P62* are discovered by WGS [40]. Briefly, the genes associated with HBV integration identified so far by deep sequencing include the HCC-related genes *TERT*, *CCNE1,* and *MLL4*, the non-tumor tissues specific gene *FN1,* and other genes such as *ANGPT1*, *CCNG1*, *FAS*, *MSMB*, *P62*, *PHACTR4*, *RBFOX*, *ROCK1*, *SENP5,* and *SMAD5* (Table **2**).

The C-terminal Truncated HBx Plays an Important Role in HCC

The most frequent integrated HBV sequence related to hepatocarcinogenesis is HBV X gene. HBV X gene is a fraction of HBV DNA located from nt.1374 to nt.1835. HBx protein (HBx) encoded by the X gene is a polypeptide of 154 amino acids, which express at low levels in acute and chronic infections [56]. HBx acts as a transcriptional *trans*-activator that is involved in the development of HBV-HCC. It also plays important roles in initiating and maintaining HBV replication / transcription during natural HBV infection [57], and takes part in epigenetic regulations of the HBV mini-chromosome [58, 59]. Integration of HBV DNA into the patient's genome is common in HBV-HCC, leading to the truncation of the HBV genome, particularly at the C terminus of HBx (Ct-HBx) [60]. A recent study has demonstrated that Ct-HBx influences the stability of HBx and the ability of stimulating HBV replication [61]. Recent NGS studies have shown that preferential chimeric sites on HBV genome are usually between nt.1500-nt.2000 [44, 50, 52], and a more precise chimeric site is an approximate nt.1800 region where viral enhancer and ORF replication sites are located [48]. One phenomenon needs to be emphasized is that chimeric transcripts can be observed only when HBV integration

cleaves at the 3'-end of HBx gene [48, 52, 62-64]. Ct-HBx translated by the integrated HBx gene can regulate the expression of WNT-5a after modification by the microRNAs [65, 66], resulting in the activation of cell proliferation *in vitro* and *in vivo* [67]. Compared with the full length HBx, Ct-HBx loses the inhibitory effect on cell proliferation and transformation, *via* abrogating p53-mediated apoptosis and cell proliferation [68, 69]. HBx is also involved in oxidative stress reaction. The C-terminal fragment of HBx (with 34 amino acids at the C-terminal end) is important for 8-oxoG (8-oxoguanine) formation *via* affecting reactive oxygen species (ROS) production. 8-oxoG functions as a key biomarker of oxidative DNA damage. Furthermore, the full-length HBx protein can induce mitochondrial DNA (mtDNA) damage *via* ROS production, while Ct-HBx loses its ability to induce ROS production or mtDNA damage. Although the role of Ct-HBx in the carcinogenesis and malignancy of HCC remains to be clarified, the effect of the C-terminal region of HBx on ROS production and mtDNA damage is ascertained [70]. A Ct-HBx (cleaved at around 130 amino acids of HBx gene) can enhance cell invasiveness and metastasis of HCC through C-Jun/AP-1 pathway, thus activating matrix metalloproteinase protein 10 (MMP10) [71], and influencing its binding to p53 regulators [52]. Epithelial-mesenchymal transition (EMT) is important as it promotes tumor progression and metastasis. A recent functional study based on the transcriptome sequencing of HBV-positive HCC cell lines has shown that a HBV-human fusion transcript long interspersed nuclear element 1 (HBx-LINE1) drives the migration and invasion of tumor cell lines *via* the induction of EMT [49]. Our previous clinical study has shown that the expression of Ct-HBx is more frequent in HCCs than in adjacent hepatic tissues (65.6% vs. 8.7%; $P<0.001$); furthermore, in the HBV-HCC patients who received antiviral treatment after curative surgery, the expression of Ct-HBx in adjacent hepatic tissues is significantly associated with a reduced recurrence-free survival [72]. Thus, we summarize that Ct-HBx integration in the host genome facilitates the development and progression of HCC.

HCC-ASSOCIATED NON-CODING RNAS IDENTIFIED BY RNA SEQUENCING

MicroRNA

MicroRNAs (miRNAs) are a group of functional small non-coding RNAs with a length of 18-25 nucleotides that regulate the gene expression at the transcriptional and post-transcriptional levels [7]. miRNAs function *via* base-pairing with complementary sequences within mRNA molecules, usually resulting in gene silencing by translational repression or target degradation. NGS is an effective approach to clarify the complete sequence of the miRNAs involved in the pathogenesis and development of cancers. Previous studies using PCR or microarrays have identified a series of miRNAs, such as pri-miR-371-373 [73], hsa-miR-191 [74], miR-27a, and miR-21 [75], which are associated with HBV- or HCV-related HCC. Recent studies using cloning technologies have indicated that deep sequencing is useful for detecting novel miRNAs, miRNA modifications, and miRNA compositions. Several studies using deep sequencing have shown that miR-122, miR-21 [76, 77], miR-34a [78], and miR-1323 [79] are aberrantly expressed in liver cancer, and sequencing-based miRNA clusters, rather than individual ones, and may serve as potential markers of early tumor recurrence after curative surgery [77]. Serum miRNAs including miR-25, miR-275, and let-7f can separate HCC from the controls, and serve as biomarkers for the diagnosis of HBV-HCC [79]. miRNAs can also function as tumor suppressor genes or oncogenes. A NGS study using Illumina platform has shown that miR-99a significantly inhibits tumor growth, suggesting that it is a potential tumor suppressor for HCC [80]. A study assessing the association of miRNA expression with doxorubicin (DOX) resistance has profiled that 22 miRNAs are helpful to overcome DOX resistance in HCC therapy [81]. These miRNAs identified using different platforms need to be cross-validated before further applications.

Long Non-coding RNA

Long non-coding RNAs (lncRNAs), with the length of > 200 nucleotides, have the capacity of transcribing into RNA but without protein-coding ability. LncRNAs may exert its gene-regulatory functions *via* epigenetic silencing, splicing regulation, translational control, and regulating apoptosis and cell cycle. Two models have been proposed to explain how lncRNAs regulate gene expression: first, lncRNAs directly affect the host genome *via* binding to the polycomb proteins [82]; second, lncRNAs alter gene expression *via* altering the chromatin such as antisense transcripts [83]. Alterations in their primary and secondary structure, expression levels of lncRNAs, and their cognate RNA-binding proteins may be related to human diseases. Their aberrant expression in cancers can influence cell growth, invasion, and metastasis. LncRNA-ATB, a microarray assay-identified lncRNA that can mediate TGF-β signaling, can predispose HCC patients to metastases and serve as a potential target for antimetastatic therapy [84]. An HBx down-regulated lncRNA, termed as lncRNA-Dreh, can inhibit HCC growth and metastasis *in vitro* and *in vivo* and act as a tumor suppressor for HBV-HCC [85]. Since some HCC-related lncRNAs can be detected in body fluid and are specific for HCC, they are suggested to be novel potential biomarkers for HCC diagnosis [86]. Some lncRNAs expressed in removed HCC and adjacent liver tissues can be developed as potent biomarkers with prognostic and predictive values or as novel therapeutic targets for HCC.

THE METHYLATION MODIFICATION IDENTIFIED BY DEEP SEQUENCING

Genetic diversity in individuals can explain the various phenotypes in life. However, individuals with similar DNAs such as monozygotic twins have different phenotypes [87]. One possible mechanism is epigenetic modification on genome. The phenotypes can be changed by epigenetic modification imposed by environmental exposure. Epigenetic modification models including DNA methylation, histone modification, chromatin remodeling, and aberrant non-coding RNA contribute to genetic changes that may drive cancer evolution. Actually, HCC-related somatic mutations frequently affect the critical genes that are responsible for chromatin modification [31, 34, 36]. Of various epigenetic modifications, DNA methylation is well characterized in cancers. Compared with normal tissues, DNA methyltransferases are more active in tumors [88, 89]. The roles of epi-

genetic modifications on HBV-HCC have been previously summarized [86, 90]. Epigenetic silencing of some important genes represents some mechanism of HBV-induced hepatocarcinogenesis and also reflects that HCC evolution trend can go into reverse *via* targeting reversible epigenetic modifications.

FRAMEWORK OF "CANCER EVOLUTION AND DEVELOPMENT"

Based on the current advances of HBV-HCC, we propose a theory framework of *Cancer Evolution and Development* (*Cancer Evo-Dev*) to characterize evolutionary regulation of HCC. Chronic hepatic inflammation, either clinical apparent or inapparent, promotes precancerous conditions, subsequently hepatocarcinogenesis, and HCC progression. Genetic predispositions of HLAs and other inflammatory factors may contribute to immune imbalance upon HBV infection, leading to persistent infection and chronic inflammation in liver. HBV mutations promoted by inflammation demonstrate an evolutionary process of "mutation-selection-adaptation". Inflammatory factors promote HBV mutations, at least partially, *via* activating AID and its analogues APOBECs. Insufficient immune responses in the immunocompromised hosts select the HCC-related HBV mutations during the long-term evolutionary process. Only the HBV strains/variants best adapted to the host immune system will survive and thrive in liver. HBV accumulates its mutations *via* minimizing the total number of epitopes recognized by $CD8^+$ T cells, particularly in the HBx and the pre-S1/pre-S2/S regions of HBV genome, to avoid immune clearance. These HBV mutations are selected *via* virus-immune interactions in the inflammatory microenvironment and in turn promote the development of HCC. AID and its analogues APOBECs, whose expressions and activities can be activated by proinflammatory cytokines generated during the inflammation, not only promote HBV mutagenesis but also facilitate somatic mutations [91]. The same explanations can be implicated in somatic mutation. Most of the mutated hepatocytes may be immunologically eliminated in the inflammatory microenvironment. Very small percentage of the mutated cell populations with survival potentials and specific growth advantages survive in the hostile environment, gradually evolve, and eventually become the major clones of cancer-initiating cells. During this relative long-term evolutionary process, somatic mutagenesis, viral integration/selection, and epigenetic modification are key elements driving the processes of hepatocarcinogenesis. Chronic inflammation in liver is often associated with aberrant DNA methylation. Activation of a natural kill cell-dependent innate immune response might contribute to the induction and accumulation of aberrant DNA methylation in human hepatocytes. This framework of *Cancer Evo-Dev* will be helpful in understanding the mechanisms by which chronic HBV infection causes HCC or some environmental exposures cause cancers of other histological types. Furthermore, it will be helpful in identifying the nodule molecules of inflammation-induced carcinogenesis which can be used as diagnostic, prognostic, and predictive biomarkers as well as therapeutic targets for effective interventions of HBV-HCC and possible other malignancies.

CONCLUSION AND FUTURE DIRECTION

Chronic inflammation, elicited by immune imbalance predisposed by HLAs and other immune molecules, facilitates the evolutionary process of HBV-induced hepatocarcinogenesis. Besides HBV factors including HBV mutations, somatic mutations, HBV integrations, and epigenetic modifications play key roles in HBV-induced hepatocarcinogenesis. High throughput technologies including NGS provide great opportunities for us to better understand the viral and genetic factors that drive hepatocarcinogenesis. Comprehensive functional experiments carried out in cell lines and animal models are essential to further characterize the roles of somatic mutations, HBV integrations, and epigenetic modifications on the HBV-HCC. Furthermore, well-designed epidemiological studies, especially prospective cohort studies are needed to evaluate and interpret the etiological roles of these molecules with HBV-HCC outcome in HBV-infected subjects. The theory framework of *Cancer Evo-Dev* proposed in HBV-HCC should be helpful to handle cancers of other histological types.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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LIST OF ABBREVIATIONS

AID	=	Activation-Induced Cytidine Deaminase
AML	=	Acute Myeloid Leukemia
APOBECs	=	Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptides
C-terminal	=	COOH-Terminal
Ct-HBx	=	C Terminus of HBx
DOX	=	Todoxorubicin
EMT	=	Epithelial-Mesenchymal Transition
HBV	=	Hepatitis B Virus
HBx	=	HBV X Protein
HCC	=	Hepatocellular Carcinoma
HCV	=	Hepatitis C Virus
HIVID	=	High-Throughput Viral Integration Detection
HLA	=	Human Leukocyte Antigen
LINE	=	Interspersed Nuclear Element
lncRNA	=	Long non-coding RNA

MAPK = Mitogen-activated Protein Kinase

MAPS = Massive Anchored Parallel Sequencing

miRNAs = microRNAs

MLL4 = Myeloid/lymphoid or Mixed-lineage Leukemia 4

mtDNA = Mitochondrial DNA

NGS = Next Generation Sequencing

nt. = Nucleotide

ORF = Open Reading Frame

PCR = Polymerase Chain Reaction

RNA-seq = RNA Sequencing

ROS = Reactive Oxygen Species

SINE = Short Interspersed Nuclear Elements

ssDNA = Single Stranded DNA

TERT = Telomerase Reverse Transcriptase

WES = Whole Exome Sequencing

WGS = Whole Genome Sequencing

8-oxoG = 8-oxoguanine

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