REVIEW

Genetic and epigenetic alterations in hepatitis B virus-associated hepatocellular carcinoma

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Hepatitis B virus (HBV) is a major cause of hepatocellular carcinoma (HCC). Its chronic infection can lead to chronic liver inflammation and the accumulation of genetic alterations to result in the oncogenic transformation of hepatocytes. HBV can also sensitize hepatocytes to oncogenic transformation by causing genetic and epigenetic changes of the host chromosomes. HBV DNA can insert into host chromosomes and recent large-scale whole-genome sequencing studies revealed recurrent HBV DNA integrations sites that may play important roles in the initiation of hepatocellular carcinogenesis. HBV can also cause epigenetic changes by altering the methylation status of cellular DNA, the post-translational modification of histones, and the expression of microRNAs. These changes can also lead to the eventual hepatocellular transformation. These recent findings on the genetic and epigenetic alterations of the host chromosomes induced by HBV opened a new avenue for the development of novel diagnosis and treatments for HBV-induced HCC.

KEYWORDS hepatitis B virus (HBV); hepatocellular carcinoma (HCC); genetic alterations; epigenetic regulations; HCC-associated microRNAs

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world and the second leading cause of cancer deaths (Laursen, 2014). The four major risk factors for HCC are chronic hepatitis B virus (HBV) infection, chronic hepatitis C virus (HCV) infection, alcohol consumption and the exposure to environmental toxins. Among these risk factors, HBV is the most important factor and responsible for over 50% of HCC cases worldwide (El-Serag and Rudolph, 2007).

HBV may induce HCC indirectly via the induction of chronic liver inflammation, which can cause oxidative stress and repeated liver injury and regeneration. This can lead to the accumulation of genetic alternations in hepatocytes and the eventual development of HCC (Cougot et al., 2005). HBV may also alter the redox status in hepatocytes and sensitize them to oxidative stress and environmental factors, leading to chromosomal instability and cellular transformation (Zheng et al., 2007; Na et al., 2011; Wang et al., 2012). Accumulating evidence indicated that HBV could also directly induce genetic and epigenetic alterations of host chromosomes to result in the development of HCC. These genetic and epigenetic alterations may be caused by the integration of HBV DNA, the DNA hypomethylation, the CpG island hypermethylation, and the aberrant expression of microRNAs. In this review, we will discuss recent studies on the genetic and epigenetic alterations in HCCs isolated from HBV patients and the possible roles of these alterations in HBV-induced hepatocarcinogenesis.

GENETIC ALTERATIONS IN HBV-ASSOCIATED HCC
HBV DNA integration in host chromosomes

HBV is an enveloped virus with a circular and partially double-stranded DNA genome of about 3.2 kb. After infecting hepatocytes, the HBV DNA is transported into the nucleus where it is converted to the fully double-stranded and covalently closed circular DNA (cccDNA). The HBV cccDNA is then assembled into a minichromosome by histones, and serves as the template to direct the transcription of viral mRNAs (Levrero et al., 2009). The HBV cccDNA is very stable and may persist for many years in the nucleus of infected hepatocytes. It can also be replenished and amplified by the HBV genomic DNA that has been replicated in the cytoplasm. The persistence of cccDNA in the nucleus as well as the continuous entry of the partially double-stranded viral DNA genome into the nucleus of infected hepatocytes significantly increases the probability of HBV DNA integration into the host chromosomes. The integration of HBV DNA into the host chromosomes was first reported 35 years ago when the HCC tissues isolated from HBV patients were analyzed (Brechot et al., 1980; Chakraborty et al., 1980; Edman et al., 1980). This DNA integration is not an essential step of the viral life cycle, but nevertheless it was frequently detected in HCCs isolated from HBV patients (Sung et al., 2012). It was initially thought that the HBV DNA integrated randomly into the host chromosomes of HCC (Matsubara and Tokino, 1990), but recent studies using the next-generation sequencing, which enables large-scale analysis of HBV DNA integration sites in HCC, led to the finding that there were recurrent HBV DNA insertion sites in the host chromosomes, and many HBV DNA actually integrated within or near repetitive sequences. The genetic loci of recurrent integration sites included FNI, MLL4, CCNE1, SENP5, ROCK1, ADH1B, CPS1, ESRRG, LRFN2, MYOM1, RAI1, FAR2, ITPR1, IRAK2, NAPK1, MLL2, CCNE1, TP53, CTNNB1, ARID1A, ARID2, AXIN1, LINEs, SINEs, SMAD5, PHACTR4 and RBFOX1 (Ding et al., 2012; Jiang et al., 2012; Sung et al., 2012; Li et al., 2013). Fujimoto et al. reported that 4 of 11 HBV-positive tumor tissues had HBV DNA integrating within or upstream of the human TERT (hTERT) gene (Fujimoto et al., 2012). Sung et al. reported that HBV DNA integration occurred 18 times at hTERT, 9 times at MLL4 and 4 times at CCNE1 in 81 HCC samples (Sung et al., 2012). The detection of recurrent HBV DNA integration sites in HCC chromosomes indicated a possible role of these integration events in facilitating hepatocarcinogenesis, which led to their positive selection in HCC.

HBV DNA integration may lead to the dysregulated expression of wild-type or truncated viral proteins that affect gene expressions or signaling pathways in the host cell (Kekule et al., 1990; Hai et al., 2014). HBV DNA integration sites in the viral genome are often located with a 1,800-bp region where the HBV enhancer, the X gene and the core protein gene reside (Sung et al., 2012). The integration of HBV DNA may be facilitated by the relaxed circular form of the viral genome, which has free 5' and 3' ends (Shih et al., 1987). The integrated HBV DNA may also exert \textit{cis} and \textit{trans} effects on the host genes. The \textit{cis} effects include the disruption or the induction of the host gene at or near the integration site, and the \textit{trans} effects often result from the production of a truncated viral gene product or the expression of a hybrid transcript containing virus and host DNA sequences that affect the expression of host genes at different locations (Ou and Rutter, 1985; Hai et al., 2014; Lau et al., 2014). HBV DNA integration has been shown to drive the expression of a long interspersed nuclear element (LINE1) repetitive sequence, generating a chimeric HBV X (HBx)-LINE1b transcript (Lau et al., 2014). This chimeric transcript functions as an oncogenic RNA that is capable of stimulating the Wnt/\(\beta\)-catenin signaling pathway. The knockdown of HBx-LINE1b RNA reduced the migratory and invasive abilities of hepatoma cells, whereas the transgene-driven expression of HBx-LINE1b promoted cell migration and invasion. HBx-LINE1b transgenic mice were more likely to develop HCC than the control mice when treated with the carcinogen diethylnitrosamine (Lau et al., 2014). These studies demonstrated that HBV DNA integration could play a positive role in hepatocarcinogenesis.

Genetic mutations

Systematic and comprehensive analyses of large numbers of matched tumor and non-tumor samples with the next-generation whole-genome sequencing had been used to study the molecular mechanism of hepatocarcinogenesis. These studies led to the identification of recurrent somatic mutations of genes in tumor samples, including genes that had not been previously reported. TP53 mutations had been identified in many HCC tissues isolated from HBV patients (for an example, see (Guichard et al., 2012)). Three genes, CTNNB1, AXIN1 and CDKN2A, had also been shown to be frequently mutated in HBV-associated HCC (Tao et al., 2011; Fujimoto et al., 2012; Kan et al., 2013). CTNNB1, which encodes \(\beta\)-catenin, is the most frequently mutated oncogene in HCC. CTNNB1 mutations occurred in 32.8% of HCC in one study (Fujimoto et al., 2012), and 62.5% in another study (Kan et al., 2013). The CTNNB1 mutation rate is about 12% in HBV-related HCC and lower than in HCCs caused by other risk factors (Guichard et al., 2012). The mutations in CTNNB1 can activate the Wnt/\(\beta\)-catenin pathway (Guichard et al., 2012), and have been suggested to act as a major oncogenic driver for HCC (Kan et al., 2013). AXIN1 encodes Axin1, which interacts with a number of cellular proteins including \(\beta\)-catenin. It often
CDKN2A, which encodes p16, displays point mutations and small deletions in HCCs. ARID1A, which encodes a key component of the SWI/SNF chromatin remodeling complex, was also found to be mutated in 13% of HCC specimens isolated from HBV patients in a separate study (Huang et al., 2012). If the expression of these chromatin regulators in HCC cell lines that expressed wild-type genes were suppressed by siRNA, cell proliferation, invasion and migration were enhanced, supporting the role of these loss-of-function mutations in the development of HCC (Fujimoto et al., 2012; Huang et al., 2012).

The somatic mutations of genes identified in HBV-associated HCCs may be generated due to the chronic liver inflammation in response to HBV infection, the induction of oxidative stress by HBV, the inhibition of DNA-damage repair by HBV and/or the exposure of HBV patients to environmental factors such as carcinogens (Becker et al., 1998; Zheng et al., 2007; Wang et al., 2012). These mutations in HCV-associated HCCs may be generated through similar mechanisms. Once these mutations are generated, they will facilitate the development of HCC.

**EPIGENETIC ALTERATIONS IN HBV-ASSOCIATED HCC**

Epigenetic modifications are important for regulating gene expression, and their abnormalities can lead to cellular transformation. Epigenetic modifications include DNA methylation and a wide spectrum of post-translational histone modifications. The importance of epigenetic alterations in the development of HCC is being increasingly recognized.

**DNA methylation**

Aberrant DNA methylation patterns, which can cause the alteration of gene expression profiles, have been found in many human cancers including HCC. Global DNA hypomethylation such as in repetitive sequences and transposable elements can cause chromosomal instability and mutations, and the hypermethylation typically at the clusters of CpG dinucleotides, known as CpG islands (CGIs), in the promoters of genes can result in the silencing of tumor suppressor genes (Lee et al., 2014). HBV infection could affect the methylation of p16INK4a, p21WAF1/CIP1, RASSF1A (Ras association domain family member 1), GSTP1 and CDH1 genes (Li et al., 2012). p16INK4a, p21WAF1/CIP1 and RASSF1A are involved in the cell cycle control and the suppression of their expression can lead to cellular proliferation. GSTP1 encodes glutathione-s-transferase P1, which inhibits oxidative damage to the cell and inactivates electrophilic carcinogens. CDH1 encodes E-cadherin and is involved in cell adhesion. Its loss of expression has been implicated in cancer progression and metastasis. Another report found ASPP1 and ASPP2 genes, which play important roles in apoptosis, are frequently down-regulated by DNA methylation in HBV-associated HCC (Zhao et al., 2010). By using mice grafted with human hepatocytes, Okamoto et al. also found that DNA methylation increased in human hepatocytes in a time-dependent manner during HBV infection (Okamoto et al., 2014).

Cellular DNA methylation may be altered by HBV infection via DNA methyltransferases (DNMTs). HBx has been shown to up-regulate DNMT1, DNMT3A1 and DNMT3A2 in cell cultures (Park et al., 2007). These DNMTs mediate the regional hypermethylation of tumor suppressor genes. HBx also suppresses the expression of DNMT3B to cause the global hypomethylation of satellite 2 repeat sequences (Park et al., 2007). Severe hypomethylation of intragenic CGIs was also observed in the HBx transgenic mouse liver before the development of HCC in these mice. These CGIs are normally highly methylated (mCGIs) by the DNMT3L complex and associated with active gene expressions. HBx together with histone deacetylase 1 (HDAC1) could bind to the promoter of DNMT3L to inhibit its expression, leading to the hypomethylation of mCGIs and the down-regulation of many developmental regulators that control tumorigenesis (Lee et al., 2014). Curiously, although HBx was found to up-regulate DNMT3A in cell cultures (Park et al., 2007), it inhibited the expression of DNMT3A in the mouse liver (Lee et al., 2014). The reason for this discrepancy is unclear.

**Post-translational modifications of histones**

The acetylation of histones plays an important role in cancer development and progression. The expression of histone deacetylases (HDACs) is altered in many human cancers, including HCC (Weichert, 2009). In a study of 12 paired HCC and its surrounding non-tumor liver tissues isolated from HBV patients, the expression of HDAC1 was found to be increased in 10 of the HCC tissues (Yoo et al., 2008). As the expression of HDAC1 was also higher in the liver of HBx-transgenic mice, the HBx protein was apparently sufficient for the induction of
HDAC1 expression. HDAC1 can also suppress p21<sup>WAF1/CIP1</sup> expression by interacting with a Sp1-binding site in the p21<sup>WAF1/CIP1</sup> promoter (Xie et al., 2012). The inactivation of HDAC1 induced the regression of tumor growth and caused the caspase-independent autophagic cell death. The HDAC1 inactivation selectively induced both p21<sup>WAF1/CIP1</sup> and p27<sup>Kip1</sup> expressions, and simultaneously suppressed the expression of cyclin D1 and CDK2 (Xie et al., 2012). The inactivation of HDAC1 also resulted in the hypophosphorylation of the tumor suppressor pRb, which then inactivated the E2F/DP1 transcription factor and inhibited the G1/S transition of the cell cycle. Besides HDAC1, HDAC2 and HDCA3 are also over-expressed in HCC (Wu et al., 2010).

MICRORNAS IN HBV-ASSOCIATED HCC

MicroRNAs (miRNAs) are small non-coding RNAs with a size of 19–25 nucleotides. They can regulate gene expression usually by gene silencing via translational repression or the degradation of mRNAs. There are more than 1000 miRNAs that have been identified in human cells. Some of them can be regulated by HBV and have been implicated in HBV-induced hepatocarcinogenesis. For examples, <i>miR-21</i> and the <i>miR-17-92</i> polycistron, which expresses <i>miR-17</i>, 18a, 19a/b, 20a and 92a, were found to be highly expressed in all of the HCCs isolated from HBV patients, and the suppression of their expression in HepG2 hepatoblastoma cells led to the reduction of the tumorigenicity of this cell line (Connolly et al., 2008). In another study, <i>miR-545</i> and <i>miR-374a</i>, which were encoded in the intron of the Ftx long non-coding RNA, were also found to be up-regulated in HBV-associated HCCs. These two miRNAs could be induced by either HBV or HBx in cell cultures, and could promote cell proliferation, migration and invasion (Zhao et al., 2014). Similarly, <i>miR-224</i> was found to be up-regulated in HBV-associated HCC (Gao et al., 2011; Lan et al., 2014). This microRNA could target <i>Smad</i> 4 to promote tumorigenesis (Lan et al., 2014). In contrast, <i>miR-145</i> and <i>miR-199b</i> were down-regulated in pre-malignant tumor nodules and HCCs of HBV patients and the restoration of <i>miR-145</i> expression was found to reduce the proliferation, migration and invasiveness of HepG2 and Hep3B cells (Gao et al., 2011). These studies indicated that HBV could induce hepatocarcinogenesis via the regulation of expression of microRNAs.

In addition to <i>miR-545</i> and <i>miR-374a</i> mentioned above, HBx, the regulatory protein of HBV, can also directly and indirectly regulate the expression of other microRNAs. HBx has been shown to inactivate p53, which reduces the expression level of <i>miRNA-23a</i>, <i>miRNA-34</i>, <i>miRNA-125b</i>, <i>miRNA-132</i>, <i>miRNA-148a</i>, <i>miRNA-192</i> and <i>miRNA-200</i>, and promote a more aggressive cancer phenotype (Zhang et al., 2009; Tao et al., 2011; Scisciani et al., 2012; Yang et al., 2012; Han et al., 2013; Noh et al., 2013). HBx can also down-regulate Let-7a to increase the Stat3 expression and promote cell prolifera-
tion (Wang et al., 2010). It can also down-regulate miRNA-152, which is frequently down-regulated in HBV-associated HCC. The expression level of miRNA-152 is inversely correlated with the expression level of DNMT1 (Huang et al., 2010), which, as mentioned above, methylates the promoters of many tumor suppressor genes. The over-expression of miR-152 resulted in the significant reduction of DNMT1 mRNA and protein levels, and its silencing induced the global DNA hypermethylation and increased the methylation levels of two tumor suppressor genes, GSTP1 and E-cadherin (Huang et al., 2010). HBx could also down-regulate miR-101, which targets DNMT3A, to increase DNA methylation of several tumor suppressor genes and to suppress their expression (Wei et al., 2013). The miR-101 also negatively regulates the expression of EZH2 (enhancer of zeste homolog 2), a histone lysine N-methyltransferase, in HCC (Xu et al., 2014). EZH2 methylates lysine-9 and lysine-27 of histone 3 (i.e., H3K9me and H3K27me) to repress the expression of target genes. The down-regulation of miR-101 by HBx can therefore increase the expression level of EZH2 and suppress the expression of its target genes. HBx could also up-regulate miRNA-21, which is known to be an oncogenic miRNA that targets PTEN, PDCD4 and RECK-tumor suppressor genes, to promote hepatocarcinogenesis (Liu et al., 2010).

**CONCLUSION**

HBV-associated HCC frequently contains genetic and epigenetic alterations. In this review, we summarized recent findings of these alterations. HBV DNA can integrate into host chromosomes to alter the expression of cellular genes and sensitize hepatocytes to oncogenic transformation. HBV can also induce DNA hypomethylation and hypermethylation to regulate the expression of cellular oncogenes and tumor suppressor genes. It may also affect the post-translational modification of histones and the expression of miRNAs to alter gene expression profiles in hepatocytes. These genetic and epigenetic alterations caused by HBV infection, which are illustrated in Figure 1, are believed to play important roles in the development of HCC in HBV patients and may serve as the targets for the development of novel therapeutic interventional treatments for treating HBV-associated HCC.

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