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Recent Advancement in Hepatitis B Virus, Epigenetics Alterations and Related Complications

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Abstract

Worldwide, it is estimated that more than 400 million people are currently living with chronic hepatitis B virus (HBV) infection, contributing to more than one million deaths annually as a result of liver cirrhosis and hepatocellular carcinoma (HCC). HBV DNA integrates into the cellular DNA in liver tissue of patients with chronic HBV infection and HCC. Following HBV infection, DNA methyltransferases (DNMTs) methylate any HBV DNA integrated into the human genome. This novel epigenetic mechanism enables the suppression of HBV antigens, leading to reduced viral replication. HBV is thought to induce DNA methylation via hepatitis B x (HBx) protein, which modulates cellular signalling pathways by activating DNMT 1 and 3 to benefit the virus. Activation of DNMT 1 and 3 inappropriately methylates host cellular genes including tumour suppressor genes whose disruption causes transformation of hepatocytes and hepatic malignancy. By being localised in the cytoplasm, nucleus and mitochondria of HBV-infected hepatocytes, it appears that HBx protein manages to exploit the entire body of cellular signalling pathways for viral survival and propagation. HBx protein may achieve its transcriptional transactivation action by either interacting with key genes or altering their related cellular signalling pathways or by hijacking their binding partners and taking over their roles. Although the underlying mechanisms are still unclear, processes such as cell cycle progression, calcium homeostasis, hepatic metabolism, protein ubiquitination, RNA splicing and vitamin D receptor regulation are key mechanisms that HBx protein alters to favour viral replication and cell survival. These detrimental effects would connect HBV infection to malignant transformation by inducing uncontrolled cell growth, proliferation and disrupting apoptosis.

Keywords: epigenetics alterations, viral integration, hepatitis B virus, hepatocellular carcinoma, hepatitis X antigen

1. Hepatitis B virus

Hepatitis B virus (HBV) is one of the most prevalent infections in humans and important cause of acute and chronic hepatitis. Chronic infection is defined as the presence of hepatitis B surface antigen (HBsAg) in the blood more than 6 months following initial infection. Without treatment, chronic HBV infection may result in the development of liver cirrhosis and hepatocellular carcinoma (HCC) [1–3].

HBV was first identified in the 1960s and was the first human hepatitis virus to be well characterised at a molecular level [3, 4]. Long-term inflammatory changes due to chronic hepatitis cause hepatocyte injury and the release of reactive oxygen species (ROS) and Kupffer cells activation. These produce proinflammatory and fibrogenic cytokines resulting in the recruitment of immune cells. The Kupffer cells also activate hepatic stellate cells which produce extracellular matrix proteins and cytokines. Repeating cycles of this activation and inflammation lead to cirrhosis characterised by regenerative nodules and irreversible fibrosis [2, 3, 5].

The ability of the virus to cause liver injury is associated with genetic changes that affect both viral and host DNA leading to mutations that predispose to liver injury and possible cancer. These events link chronic HBV infection with HCC. More than 80% of HCC cases arise in chronic HBV infection, strongly suggesting that HBV is an important contributor to the development of tumour [2, 3].

Possible mechanisms by which HBV infection causes HCC have been described, and these include HBV DNA integration, epigenetic alterations (change in gene expression) and aberrant transcriptional activities of HBx protein [3, 6, 7]. Nearly 90% of HBV-related HCC cases show evidence of HBV integration into the host genome [3, 8]. This is associated with genetic changes such as genomic instability, deletions and chromosomal translocations in the host cells, which may lead to accumulation of mutations and epigenetic changes with a malignant phenotype. Several contributing environmental and viral factors such as chronic tobacco smoking, alcohol consumption, aflatoxins, HBV e antigen positive status, high viral load and HBV genotype have been identified in HBV-related HCC cases and are associated with many epigenetic changes [3, 8–10].

1.1. Transmission Routes of HBV

HBV can be stable for 7 days or more on dry environmental surfaces. The two major routes of HBV transmission are horizontal and perinatal or vertical transmission. The efficient modes of transmission are blood and sexual contact with an infected person. The virus is horizontally transmissible during child to child physical contact or through contact with blood or infected toys. Horizontal transmission can also occur through body fluids such as semen and vaginal secretions. Perinatal or vertical transmission of HBV occurs through blood or secretions from an infected mother to the newborn baby during delivery. Perinatal transmission is high in mothers who are positive for hepatitis B e antigen (HBeAg) at 85–90% and lower in those who are negative for HBeAg where the rate is 5–20% [1, 3, 11–13].

1.2. Global epidemic of HBV infection

Worldwide, it is estimated that more than 400 million people are currently living with chronic HBV infection, contributing to more than one million deaths annually [1]. The prevalence of HBV infection is determined by the seroprevalence of HBsAg. HBV is highly endemic in Asia and sub-Saharan Africa with HBsAg seroprevalence rates exceeding 8% (**Figure 1**) [3, 14]. In these regions, the infection is typically acquired at birth or in early childhood. Progression to chronic HBV infection is common in these regions and is associated with prevalence rates of 30% for hepatic cirrhosis and 53% for HCC [16].

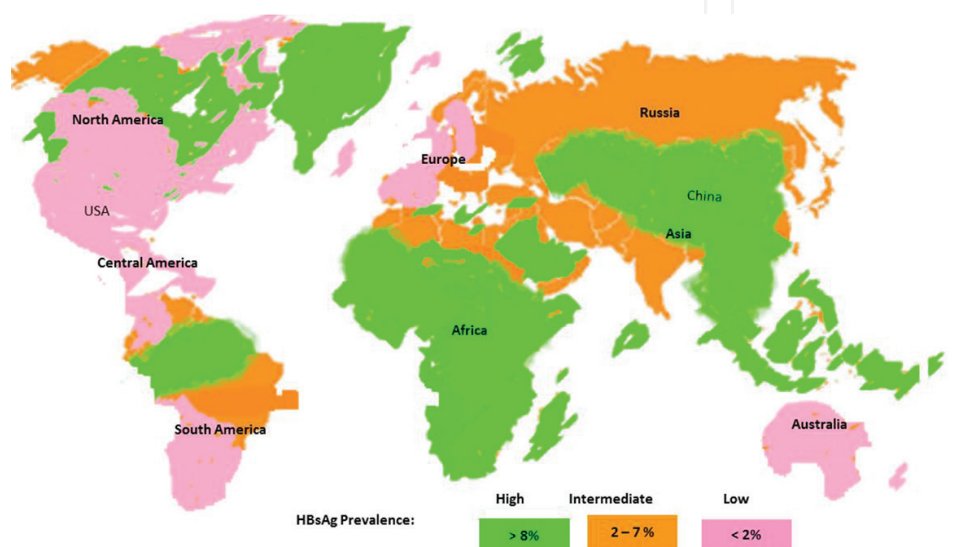


Figure 1. Global geographical distribution of chronic hepatitis B infection (Adapted from Lavanchy D [13]).

Annually, approximately one million people are diagnosed with HCC worldwide, and more than half of these people die within a year of diagnosis. Studies show that the highest HCC incidence rates of 70–80% occur in South-East Asia and sub-Saharan Africa, the regions with a high prevalence of chronic HBV infection [16]. This is due to various factors that include the late presentation of patients with large tumours, failure to recognise those at risk, high prevalence of risk factors in the population, lack of medical facilities for early diagnosis and limited access to effective treatment after diagnosis [3, 16].

An intermediate HBsAg seroprevalence of 2–7% is seen in some parts of Asia, Europe, America and Russia. The prevalence of HBV infection is low in Western Europe, Australia and United States where HBsAg seroprevalence is <2% [3, 17].

1.3. Epidemic of HBV infection in Africa

There are 65 million individuals infected with chronic HBV in Africa and 250,000 of these people die annually due to HBV-related diseases. The prevalence of chronic HBV infection in Africa varies by geographic region. It is high in sub-Saharan Africa, with HBsAg seroprevalence rates

of more than 8%. In Kenya, Sierra Leone, Zambia, Senegal and Liberia, the prevalence of HBV infection is intermediate with HBsAg seroprevalence rates ranging from 2 to 8%. North African countries including Morocco, Egypt, Algeria and Tunisia have low prevalence rates of <2%.

In South Africa and other African countries, the prevalence of HBV infection is much higher in rural compared to urban areas [18]. Low socio-economic status, infected household contact, unsafe sexual intercourse, sharing of partially eaten sweets or chewing gum, dental work and bathing towels may be some of the contributing factors for the high prevalence of HBV infection in rural areas [3, 12, 18].

1.4. HBV genotypes and genomic alterations

HBV is classified into eight genotypes (A–J) with four major serotypes (adw, adr, ayw and ayr) [3, 19, 20]. HBV genotypes are differentiated by more than 8% sequence divergence in the entire genome and more than 4% at the level of S gene. They have distinct geographical distribution as illustrated in **Table 1**. Genotype A is predominant in sub-Saharan Africa, North-West Europe and North America.

Genotype	Geographic distribution	Mutation	Host CpG promoter methylation
A	North America, Sub-Saharan Africa, North-West Europe	G1888A 1762T1764A G1862T	Induces hypomethylation and down-regulation of the <i>DLEC1</i> gene
B	Indonesia, China, Vietnam	Unknown	Unknown
C	East Asia, Korea, China, Japan, Polynesia, Vietnam	Unknown	Unknown
D	Mediterranean area, Middle East	G1896A	Induces hypermethylation and down-regulation of <i>GSTP1</i> gene
E	Africa	Unknown	Unknown
F	Central and South America, Polynesia	Unknown	Unknown
G	France, America	Unknown	Unknown
H	Mediterranean area, Middle East	Unknown	Unknown
I	South-East Asia	Unknown	Unknown
J	Japan	Unknown	Unknown

Abbreviations: A, adenine; CpG, cytosine-phosphate-guanine; *DLEC1*, deleted in lung and esophageal cancer 1; G, guanine; *GSTP1*, glutathione S transferase pi 1; HBV, hepatitis B virus; T, thymine.

Table 1. The global geographic distribution of HBV genotypes, mutations and associated CpG promoter DNA methylation.

Genotype A has four subgenotypes. Subgenotype 1A is common in South Africa, Malawi, Tanzania, Uganda, Somalia, Yemen, India, Nepal, Brazil and the Philippines [3, 20]. There

are three CpG islands within HBV genotype A, which are associated with methylation of the promoter of *Deleted in Lung and Esophageal Cancer 1 (DLEC)* gene and down-regulation of its expression in HBV-induced HCC. *DLEC* is a tumour suppressor gene and has been reported to be down-regulated in ovarian, liver, lung and EBV-related cancers [3, 21].

Genotypes B and C are more prevalent in Asia, Indonesia and Vietnam [20]. Based on the phylogenetic analysis, it was demonstrated that HBV genotype C is subdivided into 5 subgenotypes (C1–C5). Geographical clustering of these subgenotypes was clear. The subgenotype C1 was found to be prevalent in East Asia, subgenotype C2 in South-East Asia, subgenotypes C3 and C4 in Southern Pacific Ocean and subgenotype C5 in Philippines [3, 22–34]. Genotype D is commonly found in the Mediterranean region and Middle East. The hepatitis B x (HBx) protein is associated with hypermethylation and down-regulation of the *GSTP1* gene which plays an important role in the development of cancer. Genotype E is found mainly in Africa. Genotype F is found in Europe and the United States, and genotype G, in France and America. Genotype H is predominant in Central America, California and Mexico. Genotype I and J are prevalent in South-East Asia and Japan, respectively [3, 20].

HBV has a mutation rate of 10%, which is relatively high compared to other viruses. It replicates via reverse transcription of RNA intermediates that result in random mismatched base errors during genomic replication. HBV DNA polymerase lacks the ability to proofread these errors, and this predisposes HBV to mutations [3, 25]. HBV develops four major mutations which are the precore, basic core promoter, tyrosine-methionine-aspartate-aspartate (YMDD) and asparagines-to-threonine (rtN236T) mutations. The precore mutants were the first to be identified and are characterised by a nonsense G1896A mutation [3, 26]. The G1896A mutation is responsible for HBeAg negativity in chronic HBV carriers and induces the down-regulation of HLA class II molecules in hepatocytes. This mutation is common in individuals infected with HBV genotype D [3, 27]. The basic core promoter mutations include A1762T and G1764A and were identified after the precore mutations. Similar to the precore mutations, the basic core promoter mutations are found in HBeAg-negative individuals where they prevent HBeAg expression [3, 28].

1.5. Prevention and treatment

HBV infection can be prevented by avoiding direct contact with any HBV-contaminated fluids and materials. Immunisation with recombinant hepatitis B vaccines is recommended for all infants at birth and in individuals who are at high risk of acquiring the infection. Passive immunoprophylaxis with hepatitis B immunoglobulin derived from sera of positive HBV individuals is used to prevent mother-to-child HBV transmission at birth, after liver transplantation for HBV infection, needle-stick injuries and sexual intercourse [3, 20, 29].

Acute HBV infection does not require treatment as it usually resolves spontaneously. Two major classes of drugs available for treating chronic HBV infection include the injectable standard interferon- α and pegylated interferon- α_2 , and the oral nucleos(t)ide analogues. Nucleoside analogues are lamivudine, entecavir, telbivudine, whilst nucleotide analogues

are adefovirdipivoxil and tenofovir. The main aims of treatment are to improve long-term survival by reducing the risk of developing cirrhosis and HCC [3, 30, 31].

Treatment with oral nucleos(t)ide analogues is associated with the development of mutations. Lamivudine induces point mutations in the YMDD motif of the HBV polymerase, and these include rtM204V and rtM204I mutations. The viral replication rate increases in the presence of lamivudine resistance, and when lamivudine treatment is stopped, the wild-type virus reestablishes itself. Lamivudine resistance mutations are responsible for the development of resistance to entecavir that is also associated with similar mutations and more including rtI169T, rtT184G, rtS202I and rtM250V [3, 32, 33]. Telbivudine has a high antiviral potency and relatively low resistance than lamivudine and entecavir. It is associated with mutations at rtL80I/V, rtL180M, rtA181T/V, rtM204I and rtL229W/V. Telbivudine results in myopathy and neuropathy when used simultaneously with pegylated interferon- α 2, and therefore, combination of these two agents is avoided [3, 32, 34].

Adeovir treatment causes mutations that are associated with the emergence of resistant strains such as the rtN236T mutation which is downstream to the YMDD motif [35]. The use of adefovir treatment is now rare as it is associated with severe kidney injury, which may be a consequence of mitochondrial DNA depletion and activity of multidrug resistance-associated protein 4 [3, 36].

Despite the availability of treatment for chronic HBV infection, many patients will develop cancer, and this remains a major medical problem worldwide. This may be attributed to HCC-associated risk factors such as the HBV genotype, alanine aminotransferase (ALT), HBV load and HBV surface antigen level, which may influence the response to chronic HBV treatment. The response to interferon is significantly higher in patients infected with HBV genotype A compared to D and in patients with lower levels of HBV DNA and higher levels of ALT [3, 37, 38].

Aberrant methylation of promoter CpG islands is the primary epigenetic change seen during the course of HBV infection as it progresses to cirrhosis and HCC. Such methylation is detected at higher rates in HCC tissues compared to liver cirrhosis without cancer [10]. In a recent large cohort study report by Tseng et al., high HBV surface antigen levels are associated with a risk of developing HCC even in the presence of low HBV DNA levels. This finding may be due to a higher degree of viral HBV surface antigen integration into the host genome that would result in mutations and epigenetic alteration particularly DNA methylation, causing chronic liver damage, malignant transformation and HCC [3, 38–40].

The association of DNA methylation with chronic HBV treatment was first observed during telbivudine treatment. Telbivudine is a thymidine agent that interacts with protein kinases to form telbivudine 5'-triphosphate via phosphorylation. Telbivudine 5'-triphosphate competes with thymidine 5'-triphosphate, leading to the suppression of HBV DNA polymerase and reduced viral replication. Interestingly, telbivudine was recently reported to correct HBV-induced histone methylation in HBV-infected hepatocytes [3, 41].

1.6. Virological characteristics of HBV

HBV virions are infectious double-shelled particles of approximately 40–42 nanometre (nm) in diameter. They consist of a nucleocapsid core of 27 nm in diameter, which forms the inner part of enveloped virions known as Dane particles. The nucleocapsid core is surrounded by an outer surface antigen coat of ~4 nm thickness. It contains HBsAg and hepatitis B core antigen (HBcAg), which are detected in the sera of HBV-infected individuals in the form of spherical and filamentous particles [1, 3, 19, 42].

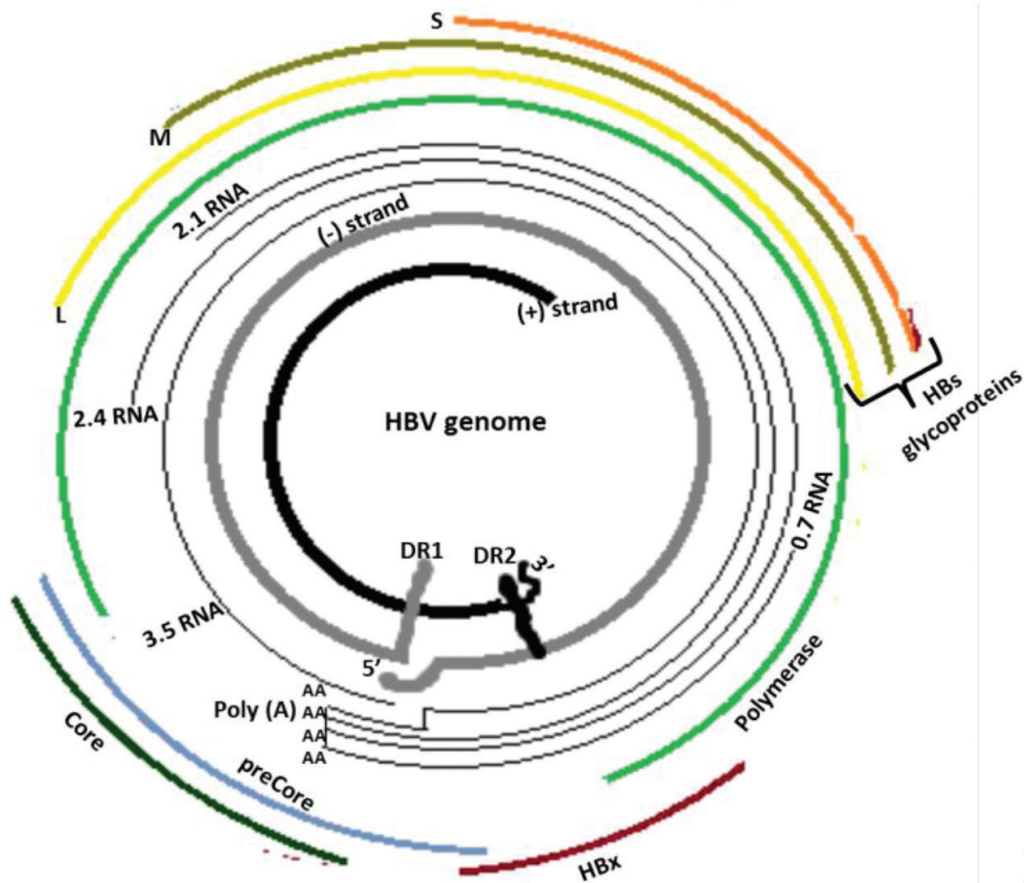


Figure 2. The structure of the HBV genome.

HBV is classified as an *Orthohepadnavirus* which belongs to the family *Hepadnaeviridae*. Contained in this family are other viruses such as the hepatic viruses of woodchucks, ducks, herons, ground and tree squirrels. These viruses replicate via reverse transcription of RNA intermediates, the step in which the DNA is packaged into hepadnaviral infectious particles. They are classified as *Hepadnaeviridaedue* to their structure and genomic organisation being similar to that of HBV. HBV genome is a small and relaxed circular molecule of 3.2 kb in size. It contains two strands of different length, a long minus strand and a short plus strand

as illustrated in **Figure 2**. The minus strand is terminally redundant and contains a second copy of direct repeat 1 (DR1), ϵ signal and poly A tail. It serves as a template for reverse transcription of a plus strand and also as a transcript for the translation of viral proteins including polymerase, HBcAg and HBeAg. The 5' end of a minus strand is covalently linked to the viral reverse transcriptase and polymerase through a phosphor-tyrosine bond. The plus strand overlaps part of the minus strand whilst its 5' end bears the oligoribonucleotides [3, 42, 43].

The HBV genome contains four ORFs, which have the same orientation and partially overlap. These ORFs encode the viral envelope pre-S/S, a pre-core/core, a polymerase and X proteins. The viral envelope also encodes three surface glycoproteins, which are the large (L), middle (M) and small (S) glycoproteins (**Figure 2**). These surface glycoproteins are synthesised by the initial transcription of pre-S/S. The L surface glycoprotein is important for viral assembly and infectivity, whilst the function of M surface glycoprotein is unknown. The longest open reading frame encodes the viral polymerase which serves as a reverse transcriptase and DNA polymerase. The pre-S/S envelope open reading frame overlaps the precore/core and X open reading frames and encodes HBsAg. The precore/core open reading frame produces HBeAg and HBcAg through cleavage by cellular proteases. HBcAg is the nucleocapsid and encloses the viral DNA [3, 11, 42, 43].

HBx protein is a transactivating protein that alters the expression of some genes via DNA methylation leading to tumourigenesis. It consists of 154 amino acid residues with a molecular weight of 27 kDa and is encoded by the smallest ORF. It stimulates viral replication either by activating viral transcription or by enhancing the reverse transcription of the viral polymerase [44, 45]. In hepatoma cell lines, HBx protein enhances viral replication by interacting with DNA binding protein 1 which interferes with cell growth and viability. In mice infected with wild-type HBV, viral replication is stimulated by HBx protein, suggesting that HBx protein is required for viral replication in normal hepatocyte cells [3, 44, 46, 47].

1.7. Life cycle of HBV

Due to the lack of efficient *in vitro* infection systems and animal models in which to study the life cycle of HBV infection, a lot of data are from the duck model infected with duck hepatitis B virus (DHBV) [3, 48]. HBV life cycle begins through the interaction of HBsAg with cellular receptor/s at the surface of hepatocytes. A number of potential cellular receptors that interact with HBsAg during HBV infection have been previously identified, but the mechanisms of action still remain controversial as none of them has been proved to be functional to HBV. These receptors include retinoid X receptor (RXR), peroxisome proliferator-activated receptor (PPAR) and farnesoid X receptor (FXR) [3, 49, 50].

Sodium taurocholate cotransporting polypeptide (NTCP) was discovered as the potential receptor for HBV infection (**Figure 2**). NTCP is abundantly expressed in the liver and is involved in the transportation and clearance of bile acids from portal blood into hepatocytes. Yan et al. [51] have shown by using near-zero-distance photo-cross-linking, tandem affinity purification and mass spectrophotometry that the pre-S/S envelope domain, a key determinant for receptor/s binding, selectively interacts with NTCP to facilitate HBV infection. Knockdown of the NTCP

expression in duck primary hepatocytes infected with DHBV significantly decreased HBV infection, suggesting that NTCP is actually required for HBV infection [3, 51, 52].

HBV requires DNA polymerase and reverse transcriptase to replicate through RNA intermediates known as pregenomic RNA. Following the interaction of surface antigen with NTCP, the viral nucleocapsid enters the host cell's nucleus to deliver dsDNA (**Figure 3**) [3, 51, 52]. In the nucleus, the dsDNA gets repaired and converted to covalently closed circular super-coiled DNA (cccDNA) by DNA polymerase. The cccDNA molecule serves as a template for the transcription of four viral RNA transcripts 3.5, 2.4, 2.1 and 0.4 kb in size, pregenomic RNA and RNA intermediate for viral replication before moving to the cytoplasm. The mRNA transcripts are then translated to produce the envelope (pre-S/S), precore/core, viral polymerase and X proteins. The 3.5 RNA transcript is reverse-transcribed into viral dsDNA [3, 8, 11, 40, 48, 53]. Some of the resulting viral DNA and polymerase-containing capsids are enveloped via budding into the endoplasmic reticulum (ER). The rest of the viral DNA is recycled or is migrated back to the nucleus where it produces new generations of cccDNA which maintains persistent HBV infection [1, 3, 11, 36, 40].

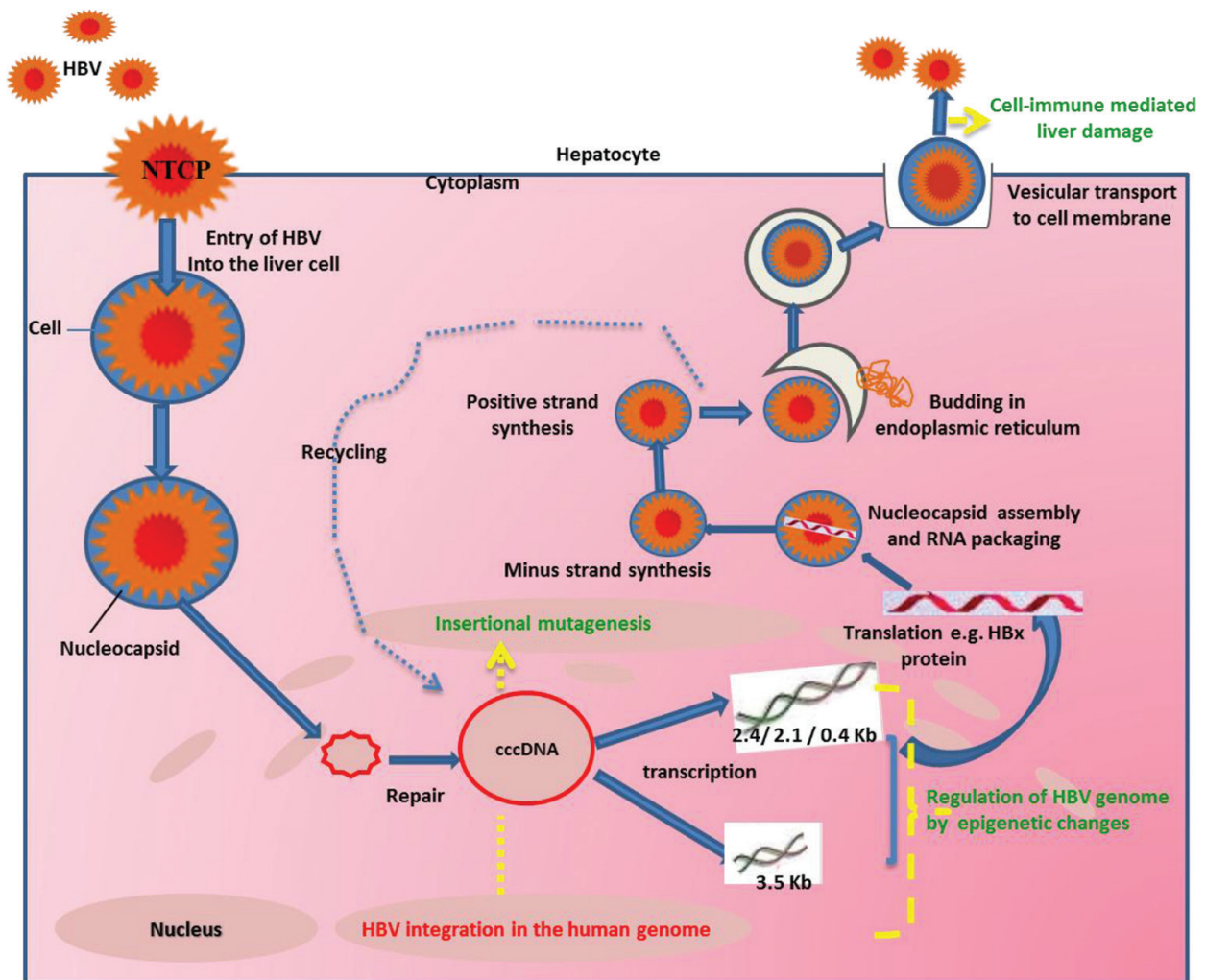


Figure 3. The life cycle of HBV infection and underlying mechanisms.

2. Epigenetics and HBV-induced hepatocarcinogenesis

Epigenetics involves attachment of chemical compounds and proteins on the DNA sequence leading to altered gene expression and normal function. There are two major ways through which gene transcription can be regulated through epigenetic changes. One way of regulating gene transcription is directly through DNA methylation. This involves the addition of a methyl group into DNA sequence. Methyl groups are carbon and hydrogen molecules which bind to the genome through the action of methyl cytosine-phosphate-guanine (CpG)-binding proteins (MeCPs), DNA methyltransferases (DNMTs), histone acetyltransferases (HATs) and histone deacetylases (HDACs), which inactivate gene transcription. Other transcription repressors including nuclear factor kappa B (NF- κ B), c-myc/c-myn, activator protein (AP)-2, E2 promoter binding factor (E2F) and cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) may also be activated by methyl groups to inhibit gene transcription [3, 53, 54].

In addition to DNA methylation, epigenetics can also be regulated by histone protein modifications. Histone protein modifications may be caused by over-expression or aberrant recruitment of HDACs that remodel the chromatin shape and structure. The two basic mechanisms responsible for chromatin remodelling are histone acetylation and deacetylation [3, 53, 55]. These mechanisms are controlled by the enzyme activity of HATs and HDACs, respectively [3, 54].

Acetylation of histone proteins is generally acknowledged as playing a key role in gene regulation. For a gene to be transcribed, it must become physically accessible to the transcriptional machinery. Acetylation by HATs substitutes the positive charges on the amino terminal tails of histone proteins with an acetyl group derived from acetyl coenzyme A, causing uncoiling of the DNA and euchromatin into an open-relaxed form of chromatin. Consequently, this makes genes accessible to several binding factors such as RNA polymerase II and transcriptional factors, allowing gene expression to occur and proteins to be made. Deacetylation of histone proteins by HDACs results in the tight coiling of the DNA and closed form of chromatin regions known as heterochromatin. This prevents the interaction between DNA and transcription factors leading to suppression of gene transcription. In some cancer cells, there is increased expression or aberrant recruitment of HDACs and decreased expression of HATs. This results in the hypoacetylation of histone proteins and therefore a condensed or closed chromatin structure [3, 54–56].

Epigenetics plays important roles in oncogenic viruses including HBV, human papillomavirus and Epstein Barr virus. In episomal HBV DNA, 3 CpG islands have been identified and described. These are island 1 located on nucleotide positions 55–286, island 2 on 1224–1667 and island 3 on 2257–2443 [57]. Methylation of CpG islands in the human genome is known to regulate gene transcription. These prompted Vivekanandan et al. [58] to hypothesise that methylation of CpG islands in HBV DNA may regulate viral gene expression. To test this hypothesis, *in vitro* methylation of the transfected HBV DNA was done, and this resulted in decreased expression of HBV mRNA and proteins in the cells. In addition, the effect of viral cccDNA methylation in the liver tissue of patients with chronic HBV infection was investigated

and found to be associated with reduced HBV replication [58]. These findings support the work of Pollicino et al. [3, 58] who showed that HBV replication is regulated by the acetylation of HBV cccDNA bound H3 and H4 histone proteins. Although these data suggest that HBV DNA methylation is a novel mechanism that influences the regulation of viral gene expression, the mechanisms of action are still not known.

Previous human studies have shown that DNA viruses integrate into the host genome and that the expression levels of DNMTs increase in response to active viral replication [59]. Vivekanandan et al. [58] hypothesised that the up-regulation of DNMTs gives infected cells the ability to methylate viral DNA and therefore control viral replication. To investigate this, the expression of DNMTs was measured in cell lines exposed to HBV DNA using two experimental systems, one of temporary transfection of cells and another that mimicked natural chronic infection. High-level expressions of DNMT 1, 2 and 3 were observed in response to persistent HBV infection. This correlated with suppressed viral replication associated with methylation of HBV DNA and increased methylation of host CpG islands [3, 58].

The seminal work of Vivekanandan et al. [58] allows for the development of a model that explains the development of liver injury and HCC in chronic HBV infection (**Figure 4**). In this model, infected host cells respond to HBV infection by up-regulating the expression of DNMTs. Up-regulation of DNMTs can also result from interaction with HBx transcriptional

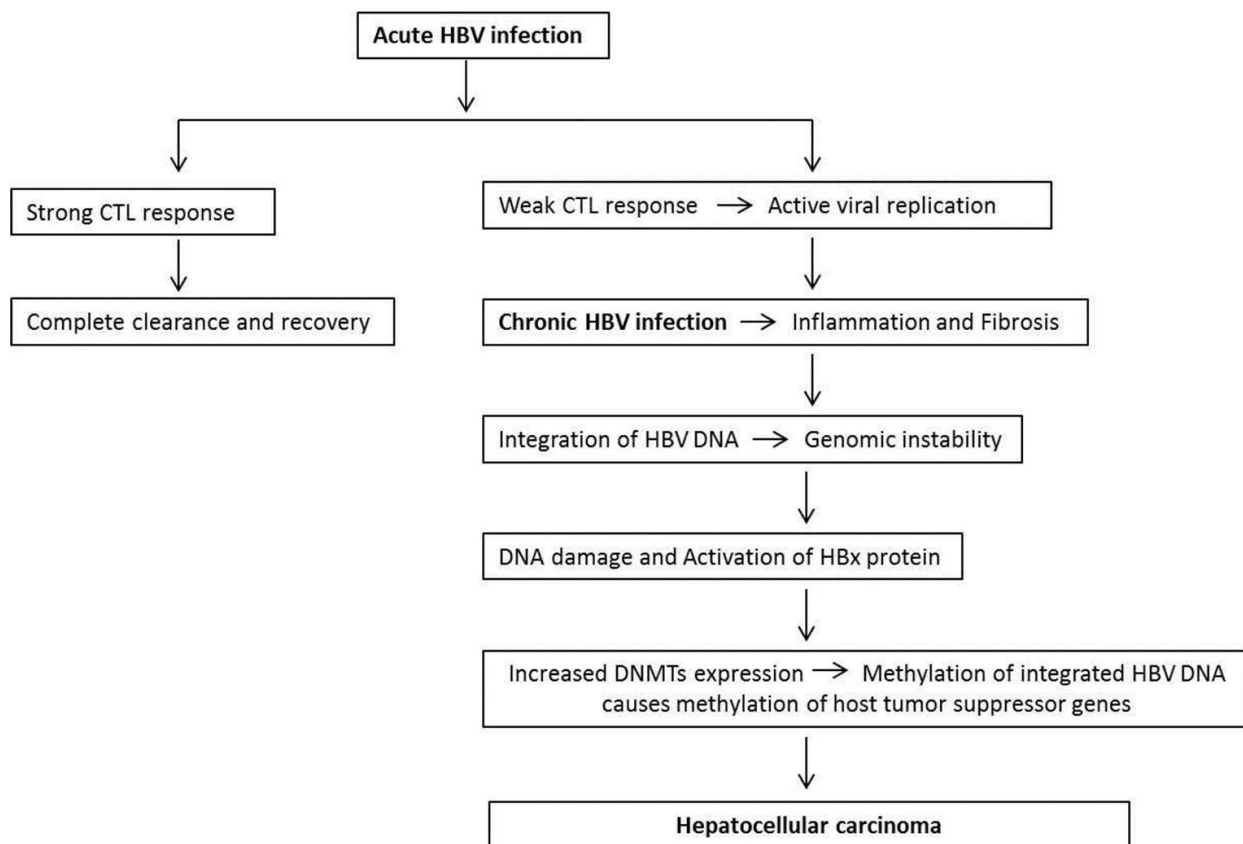


Figure 4. Model of chronic HBV infection and DNA methylation.

activator protein. Once activated, DNMTs methylate HBV DNA and switch off the expression of viral mRNA and proteins, thereby reducing viral replication. The methylation of integrated HBV DNA may be detrimental to the host genome through the inappropriate methylation of the neighbouring host genome, particularly if the promoter CpG islands regions of the gene are affected. A consequence of this effect would be the transcriptional repression of host immunoregulatory and tumour suppressor genes that prevent the development of cancer [3, 58].

Chromosomal fragile sites	Target gene	Role in tumour development
FRA1A (1p36)	TCEA; RAR; CHML	Alters gene expression and promote cell survival
FRA2C (1q)	EMX2-like gene	Modulates β -catenin signalling pathway and cell survival
FRA4E (4p)	Cyclin A	Stimulate cell cycle and anti-apoptotic effect
FRA3D (3q25.3)	IRAK2	Promotes apoptosis and tumour progression
FRA5C (5p31.1)	PDGFR β	Regulates DNA synthesis and fibrotic genes
FRA7 (7p)	SERCA 1; NCF1	β -Catenin activation
FRA9 (9q)	KLF1; CASPR3	Promote cell growth; regulates DNA methylation
FRA10A (10q)	PTEN; PI3K	Promotes metastasis; promotes cell cycle progression
FRA11A (11q13)	EMS1, FGF4; BIRC3	Modulates β -catenin signaling pathway; alters cell fate
FRA12A (12q24)	ErbB3; Mill2	Promotes tumour progression
FRA13A (13q32)	CTGF; CCNL; IMP-2	Tumour suppression
FRA18 (18q)	DCC; DPC4	Regulates methyl-CpG-binding proteins
FRA19A (19q13)	Cyclin E	Delays DNA synthesis and promotes immortalisation
FRA20 (20P12.3)	hTERT	Alters gene expression and promotes cell survival

Abbreviations: BIRC3, baculoviral IAP repeat containing 3; CASPR3, contactin-associated protein-like 3; CCNL, cyclin L1; CHML, choroideremia-like gene; CTGF, connective tissue growth factor; DCC, deleted in colorectal cancer; DPC4, deleted in pancreatic cancer 4; EMSL, EMSL; EMX2, empty spiracle homeobox 2; ErbB3, V-erb-b2 erythroblasticleukemia viral oncogene homolog 3; FGF4, fibroblast growth factor 4; FRA, fragile site; hTERT, human telomerase reverse transcriptase; IMP-2, insulin-like growth factor II mRNA binding protein 2; IRAK2, interleukin-1 receptor-associated kinase 2; KLF1, Krueppel-like factor 1; Mill2, major histocompatibility complex I like leukocyte 2; NCF1, neutrophil cytosolic factor 1; PDGFR β , platelet-derived growth factor receptor beta; PI3K, phosphatidylinositol 3 kinase; PTEN, phosphatase and tension homolog; RAR, retinoic acid receptor; SERCA, sarco/endoplasmic reticulum calcium transport ATPase; TCEA, transcription elongation factor A.

Table 2. Examples of chromosomal fragile sites associated with HBV insertions and their roles in tumour development.

HBV integrates into the host genome and promotes viral persistence. Infected cells increase the expression of DNMTs in response to viral replication. This causes methylation of HBV cccDNA and reduces viral replication. The same methylation system methylates the adjacent host tumour suppressor and immunoregulatory genes leading to hepatocarcinogenesis.

2.1. Integration of HBV DNA into the human genome

HBV integration was first discovered in 1980 using Southern blot hybridisation. It was associated with genomic instability such as loss of heterozygosity (LOH), resulting in the rearrangements, deletions, duplications and inversions of the host and viral genomic sequences. Viral integration results in the insertion of HBV DNA sequences such as HBx gene in the host genome and enables viral persistence [3, 7, 8].

Integration of HBV in the host genome also occurs in woodchucks and other animal models. In woodchucks and California ground squirrels (*Spermophilus beecheyi*), HBV genome integrates close to *ras* and *myc* family oncogenes including *c-myc*, *N-myc1* and *N-myc2*. Modulation of *myc* and *ras* family oncogenes through *cis*-activation enhances cell proliferation and transformation. These events occur via transactivation action of HBx protein and favour the development of cancer [3, 60, 61].

The occurrence of integrated HBV DNA at preferential sites in the human chromosomes has been identified using Alu-PCR-based technique. The preferential sites are known as chromosomal fragile sites (CFS) and are non-random [3, 8]. HBV DNA integrates into the human genome soon after the repair and conversion of HBV DNA to cccDNA [3, 57, 58, 62]. The HBV genome integrates within the coding sequence or close to an array of key regulatory cellular genes that can deregulate proto-oncogenes and tumour suppressor genes. Activation or inactivation of such genes promotes genomic chromosomal instability by altering various cellular signalling pathways, triggering genetic mutations and epigenetic alteration. Mutagenesis and epigenetic alteration result in the abnormal regulation of the targeted genes. This promotes malignant transformation by altering the control of cell growth, differentiation, proliferation and apoptosis [3, 57, 58, 63]. The integration of HBV at or within *cyclin A* and *RAR β* genes is associated with increased protein activities and hepatocellular growth in HBV-induced HCC, suggesting that HBV integration contributes to hepatocytes transformation [60]. Examples of known active CFS targeted by HBV integration are outlined in **Table 2**. The 60s ribosomal protein, *hTERT*, *major histocompatibility complex I like leukocyte (Mill)*, *platelet-derived growth factor receptor (PDGFR)* and *calcium signalling-related* genes are also common sites or targets of HBV integration. These genes are important in cellular signalling pathways that control DNA damage, oxidation stress and cell growth, and their alteration is associated with the development and progression of cancer [3, 9, 64].

2.2. HBx protein and its carcinogenic effects

HBx protein is a transcriptional transactivator that HBV uses to integrate into the host cellular DNA and is associated with malignant transformation in hepatocytes. It interacts with nuclear transcription factors such as NF- κ B, AP1, CREB, TATA-binding protein (TBP), per-

oxisome proliferator-activated receptor γ (PPAR γ) and transcription factor II H (TFIIH) [44]. Interaction of HBx protein with these transcription factors disrupts multiple cellular signalling pathways that include janus kinase 1 (JAK1)-signal transducer activator of transcription (STAT), mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and p53 signalling pathways. Cellular signalling pathways are important in regulating DNA repair, cell growth, differentiation, adhesion, proliferation and apoptosis. Although the precise mechanisms of action are still being elucidated, HBx protein has also been shown to induce methylation of important tumour suppressor genes critical in HBV-induced hepatocarcinogenesis by modulating DNMTs [3, 44, 45, 47, 63, 65, 66].

The transcriptional transactivation role of HBx protein on the transforming growth factor beta 1 (TGF- β 1) protein may be important in explaining liver inflammation and fibrosis. TGF- β 1, encoded by *TGF- β 1* gene, is a cytokine that is produced in response to liver injury by activated hepatocytes, platelets and Kupffer cells. It triggers apoptosis, cell growth and differentiation in human hepatocytes, hepatoma cell lines and transgenic mice [3, 67, 68]. It promotes the development of fibrosis and cirrhosis in chronic HBV infection and other liver-related diseases. HBx protein induces the expression of TGF- β 1 through the transactivation of *TGF- β 1* gene, the down-regulation of α_2 -macroglobulin and the induction of TGF- β 1 mediator Smad4. High levels of TGF- β 1 protein are observed in the sera of chronic HBV-induced HCC patients and correlate with the mutation and loss of mannose-6-phosphate/IGF-II receptor that mediates TGF- β 1 signalling [3, 67, 69, 70]. In addition, HBx protein alters the signalling pathway of TGF- β 1 from being tumour suppressive to oncogenic in early chronic HBV infection. This occurs via the activation of c-Jun N-terminal kinase (JNK) which shifts epithelial tumour suppressive pSmad3C signal to mesenchymal oncogenic pSmadL signal pathway [3, 70].

Studies show that in HBx transgenic mice and hepatoma cell lines, HBx protein can transactivate the NF- κ B, MAPK/ERK, STAT3 and PI3K/Akt cellular signalling pathways by inducing the production of ROS. Accumulation of ROS in human cancers is associated with anti-apoptotic activity, DNA damage and mutations which promote malignant transformation. HBx-induced ROS and 8-oxoguanine alter the expression of PTEN protein by oxidising cysteine residues within the promoter region encoding *PTEN* gene, which activates Akt pathway and contributes to hepatocarcinogenesis [3, 65, 70–72].

2.3. HBx protein and DNA methylation

HBx protein has been labelled an epigenetic deregulating agent. It uses its oncogenic ability to induce promoter methylation of some cellular tumour suppressor genes that contribute to the development of liver cancer [3, 73]. Cancer-associated DNA methylation may be global hypomethylation (less methylation) or hypermethylation (increased methylation). Abnormal hypermethylation of various cellular genes including host tumour suppressors has been described in liver cancer, and it is associated with silencing of genes critical for preventing malignant transformation [3, 56]. Altered gene expression has been reported in HBV infection where the DNA methylation machinery is induced as a host defence mechanism to suppress viral genes [3, 53, 57, 58]. This correlates with loss of normal activity in genes important for wound healing and immune processes. Disruption of these processes will

interfere with normal cell proliferation and apoptosis and potentiates the ability to metastasize in abnormal cells as seen in chronic liver disease and malignant transformation [3, 58, 63]. By modulating the transcriptional activation of DNMTs, HBx protein induces the hypermethylation of tumour suppressor gene promoters and silences their expression [3, 74–77].

HBx protein induces the hypermethylation of *RARβ2* gene by up-regulating DNMT1 and 3A activities and down-regulating the expression of *RARβ2* protein [3, 73, 77]. *RARβ2* binds to and inactivates the E2F1 transcription factor, which is essential for cell cycle progression [3, 64, 73, 77]. Down-regulation of *RARβ2* protein expression is associated with activation of E2F1 transcription factor, which abolishes the ability of retinoic acid to regulate the expression of G₁ checkpoint regulators, leading to up-regulation of p16, p21 and p27 proteins. The activation of E2F1 transcription factor is associated with uncontrolled cell proliferation which contributes to carcinogenesis [3, 77].

Insulin-like growth factor binding 3 (IGFBP-3) is another potential tumour suppressor gene which is both hyper- and hypomethylated in HBV-induced HCC. Hypermethylation of *IGFBP-3* gene is mediated by DNMT 1 and 3A which are upregulated via the transcriptional activities of HBx protein, and this is associated with loss of *IGFBP-3* gene expression. In contrast, HBx protein reduces the transcriptional activities of DNMT 3B, leading to hypomethylation and up-regulation of the *IGFBP-3* gene [3, 45].

DLEC1 is a functional tumour suppressor gene silenced by promoter methylation in lung, gastric, colon and nasopharyngeal cancers. Similar methylation has also been observed in HCC where it is associated with induction of G1 cell cycle arrest and loss of gene expression. Silencing of *DLEC1* gene expression is mediated by both DNA hypermethylation and histone acetylation [3, 21, 78]. HBx protein encoded by HBV genotype A enhances the transcription of *DLEC1* gene by increasing the level of histone acetylation through the activation of HATs, leading to suppression of tumour progression. Through the activation of DNMT1 expression mediated by the pRB-E2F pathway, HBx protein induces DNA hypermethylation of *DLEC1* gene and suppresses its transcriptional activities [3, 78].

Caveolin-1, encoded by *caveolin-1* gene, is an integral membrane protein abundantly expressed in adipose, fibrous and endothelial tissue. High-level expression of caveolin-1 protein disrupts growth factor signalling pathways, which in turn alters cell growth, proliferation and differentiation. HCC cells expressing high levels of caveolin-1 are associated with uncontrolled cell growth, motility, in vivo tumour aggressiveness and metastasis. Conversely, HBx-induced methylation of *Caveolin-1* gene promoter region suppresses its transcriptional activities, and this correlates with reduced tumour aggressiveness and metastasis, indicating a role of DNA methylation in HBV-related HCC [3, 80, 81].

Hypermethylation of *p16^{ink4a}* gene is a frequent event in several malignancies including HBV-induced HCC. HBx protein silences the expression of *p16^{ink4a}* gene through the activation of DNA methyltransferase 1 and the cyclin D1-CDK 4/6-pRb-E2F1 pathway. Methylation of *p16^{ink4a}* gene is associated with increased viral replication, integration and loss of protein expression [3, 80, 81].

HBx-protein-induced DNA hypermethylation has also been connected with loss of expression and normal function of *LINE-1*, *pRB*, *ASPP*, *E-cadherin*, *GSTP1* and *hTERT* tumour suppressor

genes [3, 76, 78, 82, 83]. This methylation is associated with increased up-regulation of DNMTs with DNMT1 being the most active one. Aberrant methylation of these genes is associated with perturbed cellular signalling pathways such as ubiquitination, DNA repair, transcription, proliferation and apoptosis, which may lead to the development of HBV-related HCC [3, 21, 45, 78].

Genome-wide studies aided in identifying DNA methylation, histone modifications and miRNA expression profiling across the entire samples with CHB and HBV-related HCC [3, 84–86]. Preliminary data conducted by Kgatle et al. [84] demonstrate that HBV-induced methylation may affect cellular processes such as cell cycle progression, calcium homeostasis, hepatic metabolism, protein ubiquitination, RNA splicing and vitamin D receptor regulation, which are key mechanisms that HBx protein alters to favour viral replication and cell survival. Disruption in these cellular processes could cause genetic instability, hepatocyte transformation and tumour development. However, amongst most conducted genome-wide studies, there are some discrepancies and data variations due to lack of proper normal control, heterogeneity of disease, variations of samples source, use of different technologies for analysis and validation with gene expression analysis, suggesting need for further validations [3, 84].

3. Summary

Substantial data show that there is an association between the methylation of CpG islands and transcriptional changes in gene promoter regions. Transcriptional alterations within gene promoter regions interfere with the normal function of a wide spectrum of cellular genes including tumour suppressor genes which are potential inducers of malignancies. Oncogenic viruses integrate themselves into the human genome and alter gene transcription through DNA methylation. During HBV infection, the expression levels of DNMTs are elevated in response to viral replication as viral genes are methylated to suppress viral replication. This may result in inappropriate random methylation of neighbouring host cellular genes, including tumour suppressor genes. This would cause malignant transformation and ultimately liver cancer. In addition, other genes affected by methylation may contribute to the development of liver inflammation, fibrosis and cirrhosis. As a multifunctional viral transactivator, the HBx protein may be the driving force behind the activation of DNMTs, causing gene promoter hypermethylation and gene silencing. The epigenetic alteration of genes may affect cellular signalling pathways and favour uncontrolled hepatocyte proliferation and HBV-induced inflammation, fibrosis and cancer.

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