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Genomic instability caused by hepatitis B virus: into the hepatoma inferno

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1. ABSTRACT

Chronic hepatitis B virus (HBV) infection is an important cause of hepatocellular carcinoma (HCC) worldwide, especially in Asia. HBV induces HCC through multiple oncogenic pathways. Hepatitis-induced hepatocyte inflammation and regeneration stimulates cell proliferation. The interplay between the viral and host factors activates oncogenic signaling pathways and triggers cell transformation. In this review, we summarize previous studies, which reported that HBV induces host genomic instability and that HBV-induced genomic instability is a significant factor that accelerates carcinogenesis. The various types of genomic changes in HBV-induced HCC—chromosomal instability, telomere attrition, and gene-level mutations—are reviewed. In addition, the two viral factors, HBx and the pre-S_2 mutant large surface antigen, are discussed for their roles in promoting genomic instability as their main features as viral oncoproteins.

2. INTRODUCTION

Hepatitis B virus (HBV) has been long known as an oncogenic virus for hepatocellular carcinoma (HCC). Numerous epidemiological studies in different world areas have consistently found a strong correlation between HBV infection and HCC (1-8). Because of that, for decades, the molecular mechanisms for HBV-induced HCC have been extensively investigated (9-21). HBV induces HCC through two main paths: liver inflammation caused by viral hepatitis, and the pathogen-host interplay mediated by the HBV proteins (9-21). Viral hepatitis induces the release of proinflammatory cytokines as a result of immune responses; this causes the generation of reactive oxygen species (ROS) and hepatotoxicity (22-25). Viral HBx protein and surface protein interact with host factors in DNA repair, cell cycle checkpoints, and signal transduction, and consequently lead to cell proliferation and transformation (26-33). Suffice it to say that the carcinogenic mechanisms for HBV-related HCC

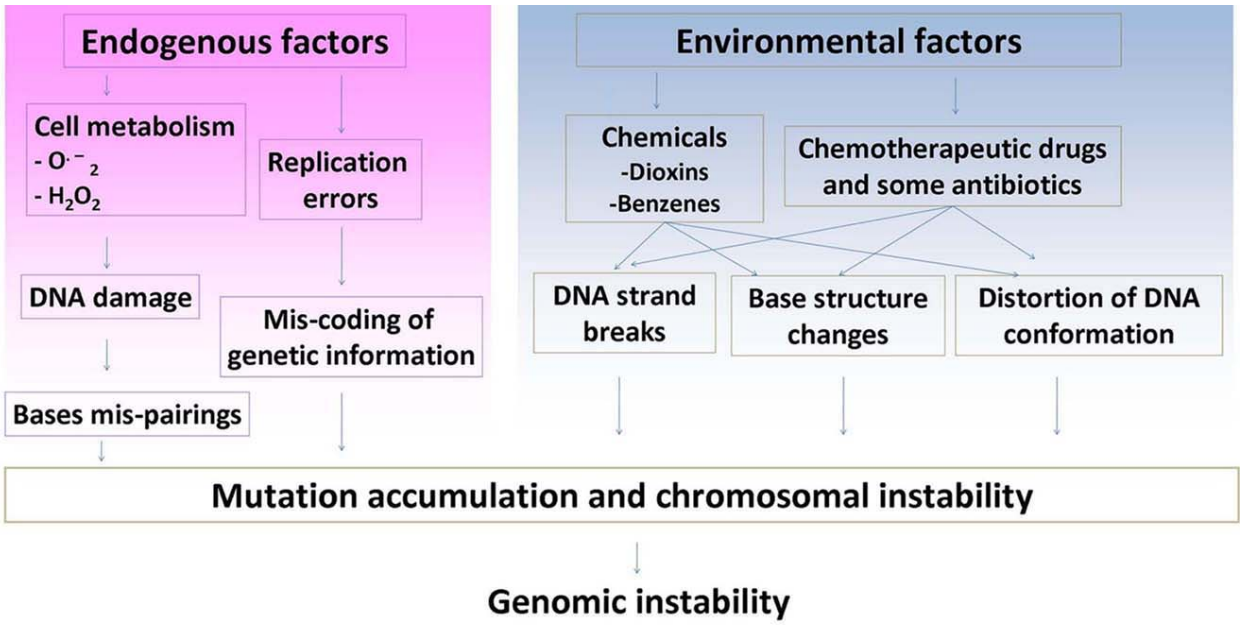

Figure 1. The basis of genomic instability.

are complicated and cannot be fully elaborated unless multiple pathways are discussed. Our recent study (90, 91, 113-119) on the HBV pre-S mutant large surface antigen found that it causes endoplasmic reticulum stress and results in strong oxidative stress and genomic instability, which establishes a new role for HBV in hepatocellular carcinogenesis. Taking together the research findings in our laboratory and others', this review summarizes the various pathways through which HBV induces genomic instability and thereby promotes liver carcinogenesis.

3. BASIS OF GENOMIC INSTABILITY

The human genome is threatened by constant genomic insult from endogenous and environmental factors. Endogenous or so-called "spontaneous" genomic insult, generated in natural processes of cell metabolism, impairs chromosomal integrity and changes nucleotide conformation. Lindahl and Nyberg (34) reported that about 18,000 purine/pyrimidine residues are lost in each cell every day by hydrolysis of the bond connecting the nitrogen base and the phosphate backbone of DNA, and Frederico *et al.* (35) showed that the transformation of cytosine to uracil residues by spontaneous deamination occurs 100 to 500 times per day in each human cell. In addition, oxygen free radicals, frequent by-products of metabolism, have been found to react readily with DNA to change or destroy the hydrogen bond affinities of individual bases and thereby cause their mispairings (36-39). Another common cause of spontaneous genetic change is base mismatch due to replication errors of DNA polymerases, which results in the miscoding of genetic information (40). Together, these endogenous and inevitable factors constantly induce changes in the chemical characteristics of nitrogen bases on genes and jeopardize the genetic integrity of each human cell.

In addition to these endogenous factors, so-called "induced" and "environmental" sources that cause DNA damage commonly exist. The DNA-reactive chemicals generated by environmental pollutant substances such as dioxins and benzenes were found to change base structure or break DNA strands (41). The chemicals used in some medicines, such as chemotherapeutic drugs and some antibiotics, tend to crosslink with DNA and distort DNA conformation (42-46). The liver is the primary organ exposed to most chemical toxins, because it is the primary organ in which most toxin metabolism occurs and ultimate DNA damaging metabolites are generated (47). An example of chemical toxin-induced DNA damage is aflatoxins, the dangerous mycotoxins produced by the fungi *Aspergillus*, which are often present in contaminated cereal grains (48). Aflatoxins are among the most potent liver carcinogens known (48). Benzo[a]pyrene, which is found in gasoline engine emissions, tobacco smoke, and grilled food, is transformed into the electrophilic metabolite benzo[a]pyrene-7,8-diol-9,10-oxide by the enzymatic activities of cytochrome p450 complexes in the liver and becomes a potent DNA damaging agent and carcinogen (49, 50) (Figure 1).

4. METABOLISM OF GENOTOXINS IN LIVER

The liver is the primary organ exposed to most endogenous and environmental genotoxins and is where these genotoxins are metabolized and transformed (47). Cytochrome p450 enzymes in the liver are crucial for activating most proximate genotoxins, i.e., xenobiotics, to toxic or tumorigenic metabolites (51). The xenobiotic biotransforming reactions by the cytochrome P450 enzymes are generally divided into two groups, called phase I and phase II (47, 51). Phase I reactions involve hydrolysis, reduction, and oxidation of xenobiotics (52-54). These

reactions expose or introduce a functional group (-OH, -NH_2, -SH, or -COOH) and usually increase their reactivities to DNA, which thereby increases their genotoxicity (55). The functional groups exposed or introduced during phase I biotransformation are often sites of phase II biotransformation. Phase II biotransformation reactions include glucuronidation, sulfation, acetylation, methylation, and conjugation with glutathione and amino acids (47). They are also believed to greatly promote the excretion of foreign chemicals and to be important for the detoxification of xenobiotics (47). Thus, through these biotransformation reactions, some xenobiotic genotoxins such as benzo[a]pyrene and carbon tetrachloride become hepatotoxic due to their activation to reactive metabolites in the liver (56). Doolittle *et al.* (57) reported that the liver injury caused by hepatotoxicity induces extensive generation of reactive free radicals, which are prone to react with DNA and cause oxidative DNA damage and DNA breaks, which lead to genomic instability (57).

Liver injury caused by inflammatory and immune responses, i.e., hepatitis, is also a major route for genomic instability in hepatocytes (58). Proinflammatory hepatitis presents common features of leukocyte migration into the regions of damaged liver, which induces the release of inflammatory cytokines and thereby augments cytotoxic effects. The proinflammatory cytokines also stimulate the generation of ROS, which attack DNA and cause oxidative DNA damage and gene mutations (59). It is worth noting that the viral hepatitis caused by HBV and hepatitis C virus (HCV) infections is among the most important causes of chronic liver inflammation and is the liver disease most highly associated with HCC worldwide (11). Therefore, it is conceivable that HBV and HCV infections are major contributors to the hepatitis-associated genomic instability in HCC. In this review, we will focus primarily on the discussion of genomic instability caused by HBV factors with respect to their contribution to hepatocellular carcinogenesis.

5. GENOMIC INSTABILITY IN LIVER CARCINOGENESIS

Genomic instability is a prerequisite for cancer development. Genomic instability caused by DNA damage or deficient DNA repair results in the accumulation of DNA mutations and, consequently, defects in gene functions (60). In addition, dysfunctional cell cycle checkpoints or apoptotic factors allow cell proliferation in the presence of DNA lesions, and make cells vulnerable to genomic changes caused by random mutations (60). Because of genomic instability, the cells are susceptible to mutations in genes associated with proliferation, differentiation, and other pro-oncogenic pathways, and eventually step into the path of oncogenesis. It has been reported (61, 62) that genomically unstable cells develop into cancer in a "fast" mode instead of "slow" mode, which indicates that random hitting for gene mutations indeed accelerates carcinogenesis. Genomic instability can be categorized into chromosomal instability (CIN), which indicates a large chromosomal change detectable in chromosomal karyotypes, and small deletions, insertions, or point mutations, which are usually undetectable unless gene-level analysis is used. The common types of CIN reported in HCC are translocations or partial deletions of a chromosome, which often lead to dysregulation of gene expression or a loss of heterozygosity due to that one gene copy is lost through chromosomal deletion (63). A cytogenetic study (64) found that HCC presents multiple chromosomal aberrations, particularly in chromosomes 1, 7, 8, 16, and 17. The loss of 1p, 4q, 6q, 8p, 9p, 10q, 13q, 16q, and 17p, and the gain of 1q, 6p, 8q, 17q, and 20q have also been recurrently reported (65-67). Nishida *et al.* (68) reported that the loss of 1p is frequently identified in well-differentiated HCCs and also detected even in dysplastic and cirrhotic nodules. It is noteworthy that the loss of 4q and 16q has been reported (68) to occur preferentially in HBV-related HCC, which suggests that HBV factors likely induce site-specific chromosomal aberrations through which hepatocarcinogenesis is promoted. Thus, these findings strongly support the high association of CIN with HCC.

Because of DNA breakages attributed to DNA repair defects, the inter-chromosomal telomere fusions caused by telomere attrition are also considered an important and direct cause of genomic instability in HCC (61, 69). DNA double-stranded breaks in telomeric regions cause uncapping of telomeres and activate telomere-telomere fusions, because all telomeres are composed of identical 6-nucleotide sequences. This so-called "breakage-bridge-fusion" cycle activates numerous interchromosomal telomere fusions, and consequently causes chromosomal non-disjunction in mitosis and aneuploidy, a common feature observed in tumor cells. A study by Plentz *et al.* (70), reporting the high prevalence of shortened telomeres in hepatocytes in cirrhosis and HCC stages, supports the telomere hypothesis of cancer initiation, which indicates that telomere breakage and uncapping initiates cancer by inducing CIN.

In addition to CIN, DNA damage caused by chemical genotoxins or hepatitis viruses induces changes of genetic information in small DNA regions, such as single or small nucleotide changes involving base substitutions, deletions, or insertions of one or a few nucleotides (60). These gene mutations alter the amino acid composition and, presumably, the function of a protein; however, they cannot be detected through cytogenetic analysis. Instead, functional and sequence analyses of genomically integrated reporter genes to estimate the overall cellular mutation frequency and spectrum have been more commonly used (71). Increased mutation frequencies in HCC have been described by a number of studies (67). These increases were mostly associated with the loss of functions in factors essential for maintaining genomic stability, such as tumor suppressor p53 and RB, and cell cycle checkpoints p16, cyclin A, and $p27^{Kip1}$, which indicates that the defects in factors in charge of maintaining genomic fidelity are important initiators for hepatocarcinogenesis (72-74).

Currently, the most accepted method for detecting genomic instability is microsatellite instability (MSI) analysis, which measures variations in DNA sizes of 5 to 7 di- or mono-nucleotide-repeat microsatellite markers in matching healthy and tumor cells. These tandem-repeat microsatellite markers are highly prone to replicational slippage, which causes insertions and deletions. The length changes of these markers are sensitively detectable using a fluorescent polymerase chain reaction (PCR) and then a GeneScan analysis of the PCR products. Although MSI offers a sensitive index for genomic instability, it is also the best recognized marker for defects in DNA mismatch repair (75). Some reports (76-78) showed that HCC with HBV infection presented MSI phenotypes in 10 to 32% of tumors; however, DNA mismatch repair defects have not been frequently found in HCC. Therefore, HBV factors are believed to somehow confer genomic instability independently of the DNA mismatch repair pathway. In the latter sections, HBV viral protein HBx and the pre-S2 mutant large surface antigen will be discussed with respect to their induction of genomic instability in host cells.

6. ASSOCIATION OF HEPATITIS B VIRUS INFECTION WITH HOST GENOME INSTABILITY

Recent studies (79) have demonstrated that HCCs associated with chronic HBV infection display more allelic deletions and amplifications than those not associated with HBV. HBV belongs to the family of hepadna viruses and is a small, enveloped, partially double-stranded DNA virus. With a size of 3.2 kilobase (kb) pairs, the HBV genome is one of the smallest animal virus genomes and is compactly organized (80). The HBV genome encodes four translated products: surface, polymerase, core, and X proteins. These viral proteins are essential for viral genome replication, Dane particle assembly, and infection (80). During viral infection the virus-specific cytotoxic T cells recognize viral antigens presented on infected hepatocytes and lead to either the direct lysis of the infected hepatocytes or the release of interferon (IFN)-γ and TNF-α, which downregulate viral replication in surrounding hepatocytes (59, 81-84). Thus, these cytokines are involved both in viral clearance and in inflammatory tissue damage mechanisms (85). IFN-γ and TNF-α stimulate the production of oxygen free radicals, which contribute to liver injury (86). They also activate secondary messengers to further activate the signals for ROS production, e.g., expression of inducible nitric oxide synthase (iNOS) (87). These primary and secondary reactive oxygen free radicals directly attack cellular DNA, which induces oxidative DNA lesions and, consequently, increases gene mutations in hepatocytes (60). As an example, the most frequently generated oxidative DNA lesion, 8-hydroxyguanine, tends to mispair with adenine or thymine and results in transition and transversion mutations (88). Another common oxidative DNA lesion, thymine glycol, which is generated by the hydroxylation of C5 and C6 on thymine, stalls DNA polymerase at DNA forks and induces replication errors (89). Studies (90, 91) on HBV have found that HBV-infected hepatocytes exhibited greater oxidative stress and DNA damage than the surrounding uninfected cells, which indicates that HBV infection indeed induces genomic instability, thereby activating hepatocarcinogenesis.

In chronic HBV infection, viral DNA often integrates into the host genome, which causes persistent viral replication in the cell. Almost all HBV-associated HCCs harbor chromosomally integrated HBV DNA (92). The consequences of HBV-DNA integration are chromosomal DNA instability caused by insertional mutagenesis or cis-activation of cellular genes near the insertion sites. In the case of woodchuck hepatitis B virus (WHV)-related HCC, WHV-DNA preferentially inserts itself within or near the proto-oncogenes c-Myc or N-Myc and likely activates their expression (93). However, in HBV-associated HCC in humans, site-specific integration of the HBV genome or integration of the HBV genome into known oncogenes seems to be a rare event. Interesting examples are the integration of HBV DNA in a cyclin A gene, in the retinoic acid receptor beta gene, in the mevalonate kinase gene, or in the sarco/endoplasmic reticulum calcium ATPase 1 gene (94-97). The regulation of HBV genome integration on cellular gene functions through cis-activation is believed to be a random event. Thus, the mutagenic effects to the integrated cellular genes create aberrations of gene functions for these genes. In many cases, the integrated viral genomes are characterized by rearrangements or partial deletions, or both, because HBV integration induces deletions in the host chromosome at the integration site (95).

In addition to HBV-DNA integration's causing host genome instability, the viral factors HBx and the pre-S_2 mutant large HBV surface antigen (LHBS) also induce host genome changes through their direct associations with host factors, which causes a loss of genetic integrity. The mechanisms of genomic instability caused by HBx and pre-S_2 mutant LHBS are mentioned below:

6.1. Viral oncoprotein HBx induces cell cycle progression and inhibits DNA repair

In most integrated subviral HBV genomes, the open reading frame for HBx regulatory protein is conserved and can be transcribed. The HBx gene is conserved in all mammalian hepadna viruses. HBx is a small polypeptide (17 kDa) produced at very low levels during chronic and acute hepatitis. It is crucial in HBV transcription and replication (98-100). Early studies (101) showed that HBx stimulates the activity of viral promoters and enhancers, an effect primarily attributed to the transactivator activity of nuclear HBx protein. By directly interacting with basal transcription factors and acetyltransferase CBP/p300, HBx also functions as a transcriptional transactivator of different host genes involved in cellular proliferation control, such as c-Jun, c-Fos, and c-Myc (102). HBx upregulates G1/S and G2/M cell cycle progression by activating cyclin E/A-CDK2 complex (103).

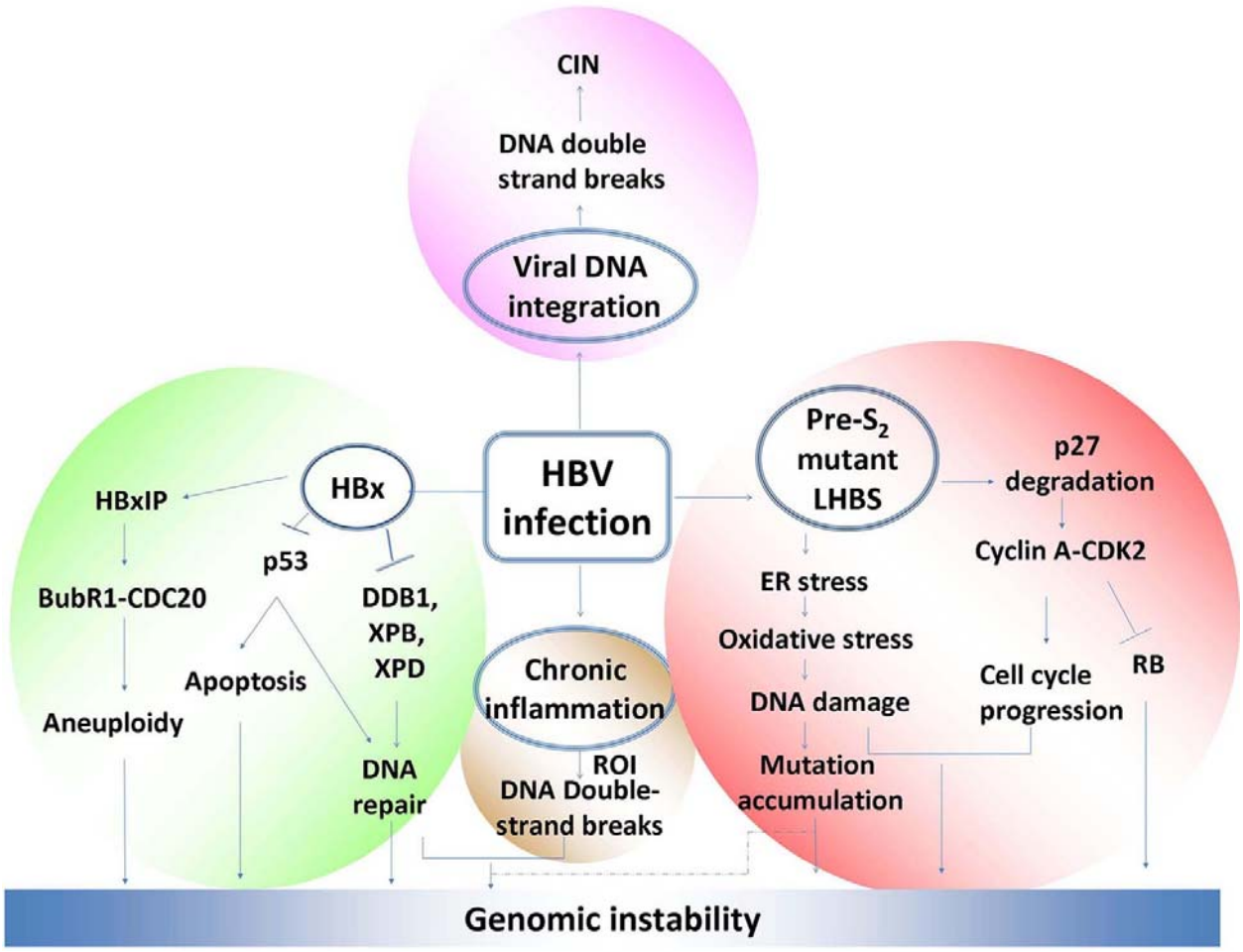

Figure 2. Genomic instability caused by HBV infection. Hepatitis-induced chronic inflammation induces the generation of reactive oxygen ions (ROI), which attacks DNA and breaks it. When viral DNA integrates into the host genome, it induces chromosomal breakage and CIN. Meanwhile, the viral factor HBx interacts with some cellular proteins involved in DNA repair, cell cycle checkpoints and apoptosis, inhibiting DNA repair activity and causing DNA mutations. In addition, the misfolded pre-S_2 mutant LHBS accumulates in ER and induces ER stress-dependent oxidative stress and DNA damage. Through interacting with JAB1 protein, it also triggers $p27^{Kip1}$ degradation and ultimately cell cycle progression. The concerted actions of these different effects lead host cells to genomic instability.

HBx is involved in cell cycle control in multiple ways. In addition to upregulating G1/S and G2/M cell cycle progression, HBx interacts with the BubR1 mitotic spindle assembly checkpoint protein and interferes with the binding of BubR1 to cell division cycle 20 (CDC20) protein, which contributes to aberrant chromosomal segregation (104). Recent studies (105, 106) also report that HBx associates with HBx-interacting protein (HBxIP) to cause excessive centrosome replication, which results in tripolar and multipolar spindles and defective cytokinesis. All these findings taken together clearly show that HBx is involved in promoting CIN induced by dysregulating the mitotic processes.

An additional HBx-induced mechanism for genomic instability is that HBx interferes with cellular DNA repair. HBx directly interacts with nucleotide excision repair (NER) protein, DNA damage-binding protein 1 (DDB1), xeroderma pigmentosum B (XPB), and XPD to inhibit NER (26, 29, 107, 108). Also, by directly binding to the tumor suppressor p53, HBx inhibits p53 transactivation activity (27, 28, 109, 110). HBx also efficiently blocks p53-dependent apoptosis and DNA repair activities in the *in vitro* cultured hepatocyte cell lines (111). In summary, HBx blocks tumor suppressive phenotypes through its pleiotropic activities on cell cycle regulation, signaling pathways, and DNA repair, and displays its pivotal role in tumor transformation. Therefore it has been designated a "viral oncoprotein" (112).

6.2. The novel pre-S_2 mutant LHBS induces endoplasmic reticulum stress-induced oxidative DNA damage and mutation

The pre-S_2 mutant LHBS is a newly identified viral oncoprotein. It contains an approximately 50-nt deletion in the pre-S_2 region of the large surface protein. In the late 1990s, the pre-S_1/S_2 mutant LHBS was identified in ground glass hepatocytes (GGH), the histological hallmark for

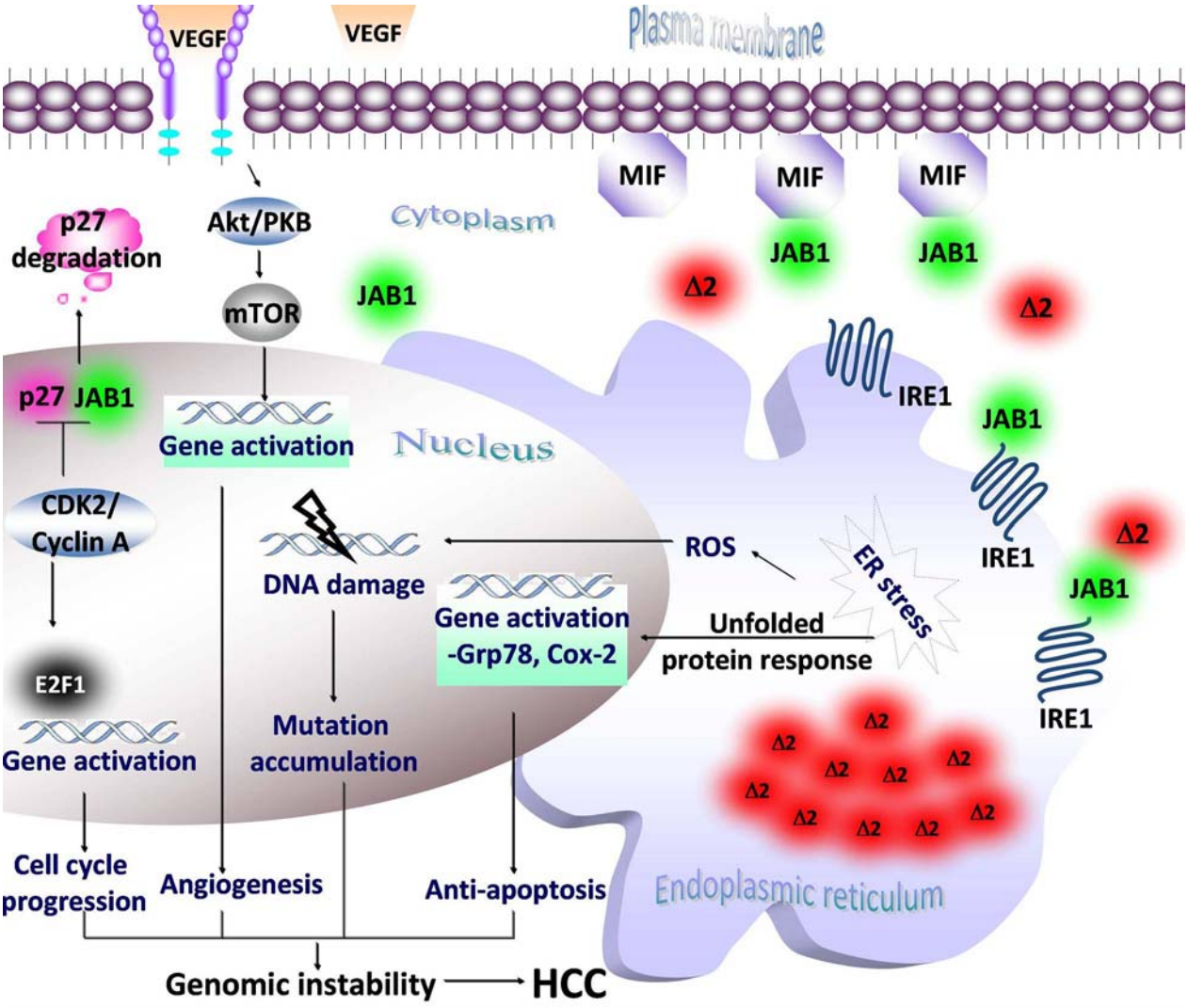

Figure 3. Summary of the carcinogenic pathways regulated by the pre-S_2 mutant LHBS. The pre-S_2 mutant LHBS (A2) accumulated in ER activates the unfolded protein response, ER stress-mediated oxidative stress, and DNA damage, all of which result in gene mutations. It also interacts with JAB1 and modifies the protein complexes of JAB1 with the factors IRE1 and MIF1. Through ER stress, the pre-S_2 mutant LHBS (A2) also induces VEGF expression, which activates the Akt/PKB pathway and, subsequently, cell proliferation. The activation of cell proliferation and cell cycle progression in the presence of DNA mutations renders cells significantly more genomically unstable and thereby promotes HCC.

HBV-induced HCC (18, 20, 113, 114). The pre-S_1/S_2 mutant LHBS contributes to two histological patterns: type I GGH and type II GGH. Type I GGH displays an inclusion-like pattern of HBS; while type II GGH displays HBS at the margins in hepatocytes. The pre-S_1 mutant LHBS in type I GGH harbors the LHBS that is partially truncated in the pre-S_1 region; however, in type II GGH, it is partially truncated in the pre-S_2 region. Some of the pre-S_2 mutant LHBS isolates contain point mutations at the start codon of the middle surface antigen and cause a dramatic decrease in its levels (113, 114). An electron microscopy study (18, 115) found that both the pre-S_1 and the pre-S_2 mutant LHBS accumulate in endoplasmic reticulum (ER) and induce strong ER stress as well as the associated signaling pathways (18, 115). The pre-S_2 mutant LHBS, particularly, is highly correlated with clonal growth advantage (116, 117). Immunohistochemical and pathological studies (18, 20, 116) found that the hepatocytes expressing the pre-S_2 mutant LHBS consistently cluster into groups due to clonal and integrated expansion, which indicates that the pre-S_2 mutant LHBS increases cell proliferation.

The pre-S_2 mutant LHBS usually emerges in chronic HBV carriers rather than in recently infected individuals (118). It is highly expressed in most of the HBV-induced cirrhotic nodules through the stages of the pre-neoplastic lesions, early HCC, and large HCC tumors, which implies its importance in tumor progression (20, 113, 114, 118). Like HBV oncoprotein HBx, the pre-S_2 mutant LHBS interacts with some host proteins through which it regulates the cellular functions related to carcinogenesis. We found (119) that the pre-S_2 mutant LHBS

specifically interacts with c-Jun activation domain-binding protein 1 (JAB1), which degrades the cyclin-dependent kinase (Cdk) inhibitor $p27^{Kip1}$, increases Cdk2 activity, and inactivates the tumor suppressor retinoblastoma protein through hyper-phosphorylation. Through transcriptional activation, it also increases the level of cyclin A in a cell (116). These effects together trigger the phenotype of cell cycle progression in pre-S_2 mutant $LHBS^{(+)}$ hepatocytes (Figure 2).

We previously reported (90) that pre-S_2 mutant LHBS induced ER stress-dependent oxidative stress and DNA damage in *in vitro* cultured hepatoma cell lines, pre-S_2 mutant LHBS transgenic mice, and type II GGH cells from human HCC patients, which indicated that pre-S_2 mutant LHBS is genotoxic. The cells expressing pre-S_2 mutant LHBS exhibit the typical phenotypes of genotoxic stress: DNA breakages and gene expression of the DNA repair factors induced in response to DNA damage. Given that the pre-S_2 mutant LHBS also induces cell cycle progression, it is conceivable that the cells continue to proliferate in the presence of unrepaired oxidative DNA damage, which makes them vulnerable to mutation accumulation and genomic instability (119). We found that in the mouse ML-1 hepatoma cell line, the pre-S_2 mutant LHBS stimulated mutations at the x-linked *hprt* gene, which supported the notion that the pre-S_2 mutant LHBS accelerates carcinogenesis by inducing genomic instability (90). Therefore, the pre-S_2 mutant LHBS is a novel viral oncoprotein that promotes hepatocellular carcinogenesis by inducing genomic (Figure 3).

7. CONCLUSIONS

Genomic instability is an essential parameter for cancer development. Just as CIN has been frequently observed in hepatoma, so, too, have the small deletions, insertions, and point mutations in the tumor suppressor genes and oncogenes been found to highly correlate with the initiation and progression of these tumors. In areas in which HCC is highly prevalent, HBV-related HCC accounts for more than 80% of HCC cases. This review summarizes the recent findings about the contributions to hepatocarcinogenesis of two HBV oncoproteins, HBx and pre-S_2 mutant LHBS. Their direct and indirect involvements in promoting host genome instability were extensively discussed. These effects are believed to be among the most important examples of virus-induced host genome change that causes tumor development. They also represent a molecular mechanism of carcinogenesis through virus-host interplay. Some other viral oncoproteins, e.g., human papillomavirus E6 protein and hepatitis C virus nonstructural 3/4A protein, have also been associated with tumorigenesis through direct interaction with host factors, which increases host genomic instability (120, 121). Therefore, this is a general mechanism for some virus-induced carcinogenic pathways. Maintaining genomic integrity in the respective virus-infected host cells by improving their DNA repair activities is a useful approach for inhibiting carcinogenesis in these cells.

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