

Hepatitis B virus pre-S mutants, endoplasmic reticulum stress and hepatocarcinogenesis

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Although hepatitis B virus (HBV) has been documented to cause hepatocellular carcinoma (HCC), the exact role of HBV in the development of HCC remains enigmatic. Several hypotheses have been proposed to explain the potential mechanism, including insertional mutagenesis of HBV genomes and transcriptional activators of HBV gene products such as hepatitis B x protein (HBx) and truncated middle S mutants. In the past few years, we have identified two types of large HBV surface antigens (LHBs) with deletions at the pre-S1 (Δ S1-LHBs) and pre-S2 (Δ S2-LHBs) regions in ground glass hepatocytes. The pre-S mutant LHBs are retained in the endoplasmic reticulum (ER) and escape from immune attack. The pre-S mutants, particularly Δ S2-LHBs, are increasingly prevalent in patients with hepatitis B e antigen (HBeAg)-positive chronic HBV infection, ranging from 6% before the 3rd decade to 35% in the 6th decade. In HCC patients, the two pre-S mutants were detected in 60% of HCC patients, in the serum and in HCC tissues. Pre-S mutant LHBs can initiate ER stress to induce oxidative DNA damage and genomic instability. Furthermore, pre-S mutant LHBs can upregulate cyclooxygenase-2 and cyclin A to induce cell cycle progression and proliferation of hepatocytes. In transgenic mice, the pre-S mutants can induce dysplasia of hepatocytes and development of HCC. In a nested control study, the presence of pre-S mutants carried a high risk of developing HCC in HBV carriers. In summary, the findings we describe in this review suggest a potential role for HBV pre-S mutants in HBV-related hepatocarcinogenesis, providing a model of viral carcinogenesis associated with ER stress. (*Cancer Sci* 2006; 97: 683–688)

Hepatitis B virus is recognized as a major etiological factor in the development of HCC.^(1,2) Epidemiological studies have demonstrated an approximately 100-fold increase in the relative risk of HCC among HBV carriers compared to non-carriers.⁽³⁾ Although the relationship between chronic HBV infection and HCC has been well established, the exact role of HBV in the pathogenesis of HBV-related hepatocarcinogenesis remains to be elucidated.

Hepatitis B virus is a partially double-stranded DNA virus containing a genome of 3.2 kb in size, which contains four open reading frames encoding viral polymerase, the core and e antigen, the HBx protein and the pre-S/S gene encoding the three surface antigens (i.e. the large [pre-S1 + pre-S2 + S],

middle [pre-S2 + S] and small [S only] surface proteins). In chronic HBV infection, HBV DNA can be integrated into the host genome. Almost all HCC harbor single or multiple copies of integrated HBV DNA.^(4,5) The integrated HBV DNA in tumors is usually rearranged and partially deleted. HBV DNA integration has been shown to be a random event and no specific *cis*-effect has been observed on flanking cellular genes.^(6,7) Therefore, HBV DNA integration *per se* is not considered to be a general mechanism of HBV-related hepatocarcinogenesis. Based on the observations obtained from several isolates of human HCC tissues, the integrated HBV DNA usually has truncated open reading frames coding for viral polymerase and the core antigen, and can only encode two gene products: the HBx and HBs proteins. Therefore, HBx and HBs proteins represent the two potential candidate proteins involved in HBV-related hepatocarcinogenesis. Although still controversial, the HBx protein has been studied extensively. It has been shown to exhibit transactivating functions, and to activate JAK/STAT and the Ras–Raf–MAPK signal pathway.^(8,9) In the case of HBs protein, the LHBs has been demonstrated to exert a tumor promoter-like function in the development of HCC.^(10,11) The C-terminally truncated middle surface protein MHBst has been recognized as a transactivator and initiates c-Raf-1/MAPK-2 signaling.^(12,13)

In past years, we identified two types of LHBs with deletions at the pre-S1 (Δ S1-LHBs) and pre-S2 (Δ S2-LHBs) regions in GGH obtained from patients with advanced diseases of chronic HBV infection. These naturally occurring pre-S mutant LHBs are increasingly prevalent in serum and liver tissues of patients with chronic HBV hepatitis and in HCC tissues. The pre-S mutant LHBs are retained in the ER and induce ER stress signals. Furthermore, the pre-S mutant LHBs can upregulate COX-2 and cyclin A to induce cell cycle progression. In transgenic mice, Δ S2-LHBs has been shown to induce dysplastic changes in hepatocytes and development of HCC. In this review, we describe the biological

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Abbreviations: COX-2, cyclooxygenase-2; ER, endoplasmic reticulum; GGH, ground glass hepatocytes; HBeAg, hepatitis B e antigen; HBs, hepatitis B surface protein; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBx, hepatitis B x protein; HCC, hepatocellular carcinoma; JAB1, Jun activation domain-binding protein 1; LHBs, large surface protein; NF κ B, nuclear factor κ B; MAPK, mitogen-activated protein kinase; RB, retinoblastoma protein; Δ S1-LHBs, LHBs with a deletion at the pre-S1 region; Δ S2-LHBs, LHB with a deletion at the pre-S2 region; UPR, unfolded protein response.

characteristics of HBV pre-S mutants and their possible roles in the development of HCC based on the data mainly from our past studies.

Identification of pre-S (LHBs) mutants with deletions at the pre-S1 and pre-S2 regions in GGH

Ground glass hepatocytes have been recognized as the histological hallmark of chronic HBV infection since 1973 when Hadziyannis *et al.* demonstrated the presence of HBV surface antigens in the 'glassy' cytoplasm of hepatocytes (Fig. 1a).^(14,15) Ultrastructurally, GGH are characterized by an abundance of ER in which HBsAg is accumulated.⁽¹⁶⁾ Several types of GGH have been recognized and correlated to the different replicative stages of chronic HBV infection.^(17–20) Of particular interest is the emergence of a so-called 'marginal type' GGH (type II GGH) at advanced stages of chronic HBV infection.⁽²⁰⁾ Marginal type GGH harbor HBsAg at the cell margin or periphery of hepatocytes, distinct from the 'inclusion-like' accumulation of HBsAg in classic GGH (type I GGH; Fig. 1b). Furthermore, marginal type GGH consistently cluster in groups or nodules in liver tissues, suggesting a clonal proliferation or growth advantage of type II GGH.

The differential expression patterns of HBsAg in GGH imply the possibility of the existence of different types of HBV surface antigens. By laser-capture microdissection, we identified two major types of LHBs or pre-S mutant LHBs with deletions at either the pre-S1 (Δ S1-LHBs) or pre-S2 (Δ S2-LHBs) region in type I and type II GGH, respectively (Fig. 1c).^(21–23) This interesting finding drives us to explore in depth the potential biological significance of GGH, particularly the role of type II GGH or Δ S2-LHBs in the pathogenesis of HBV-related tumorigenesis.

Pre-S mutants represent immune selection variants and are increasingly prevalent during the course of chronic HBV infection

The presence of different types of viral mutants in different replicative stages of chronic HBV infection suggests the potential evolution of viral variants under immune pressure during HBV infection. Naturally occurring pre-S mutants are detected frequently in serum obtained from patients with chronic HBV infection.⁽²⁴⁾ The resulting mutants reveal shorter forms of LHBs proteins with internal deletions. By examining serum samples obtained from longitudinal studies of patients with chronic hepatitis B over 10 years, we observed the sequential appearance of a wide spectrum of LHBs mutants, with the first appearance of variants with point mutations at the S region, followed by the presence of Δ S1-LHBs, and later the emergence of Δ S2-LHBs after e seroconversion (after serum HBeAg status converted from positive to negative) (W. C. Chen and I. J. Su, unpublished data). The deletion site (nucleotides 4–57) of Δ S2-LHBs has been recognized to correlate with the epitope of the CD8 T-cell response and B-cell neutralization.⁽²⁵⁾ The pre-S2 mutant Δ S2-LHBs may therefore represent an immune escape mutant that usually appears at the later stage of chronic HBV

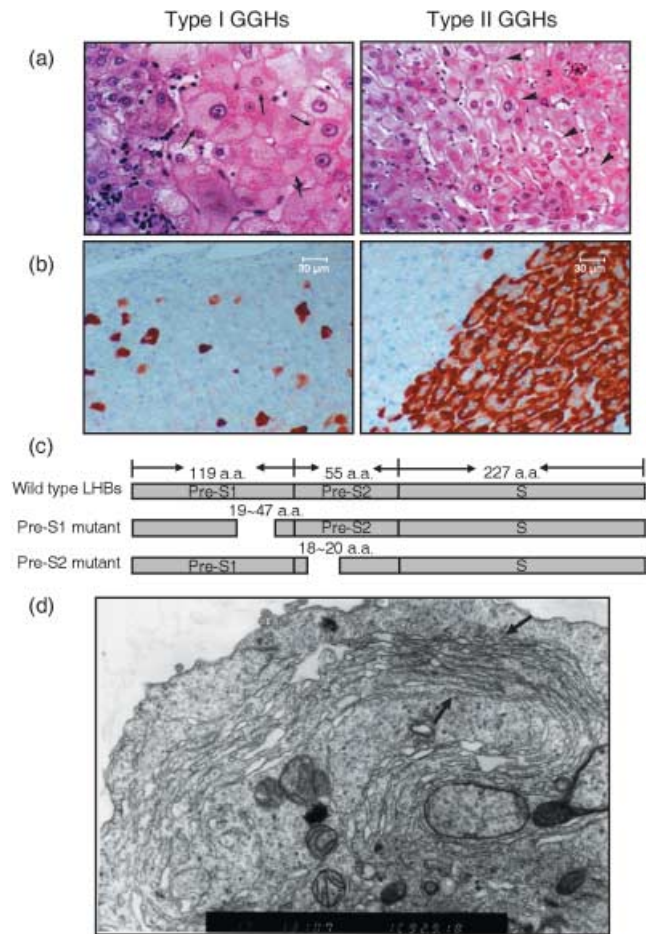


Fig. 1. Histopathological characteristics of ground glass hepatocytes (GGH). (a) Hematoxylin–eosin staining of type I and type II GGH. Black arrows indicate hepatocytes containing ground glass substances in both type I (left) and type II GGH (right). (b) Immunostaining of surface proteins on livers of patients with chronic hepatitis B virus infection. Type I GGH (left) usually contain hepatitis B surface antigen (HBsAg) in a blot-like or inclusion pattern. In contrast, HBsAg is usually expressed in the marginal region of type II GGH (right). (c) Mutation map of the identified pre-S1 and pre-S2 deletion mutants. Dotted lines indicate the deletion regions. (d) Electron microscopy of HuH-7 cells expressing large surface protein (LHBs) with a deletion at the pre-S2 region. Endoplasmic reticulum proliferation in the marginal region of the cell is indicated. (Original amplification: $\times 10\,000$.)

infection. In liver biopsy tissues of chronic HBV infection, there is usually an absence of lymphocyte infiltration in regions of type II GGH, supporting the concept of an immune escape for pre-S2 mutant Δ S2-LHBs.

We have recently observed the prevalence of pre-S mutants in patients with HBeAg-positive chronic HBV infection who received antiviral therapy. The prevalence of pre-S mutants in serum increases from around 6% at the early stage (2nd decade of age) of chronic HBV infection with high HBV viral load, to the peak level of 35% during the 5–6th decade with low titers of HBV DNA. In patients with HCC, the prevalence rate of pre-S mutants increases up to 60% in both serum samples and liver tissues. Furthermore, the Δ S2-LHBs appear to predominate over Δ S1-LHBs in serum obtained from advanced diseases and in HCC tissues.

Pre-S mutant proteins are accumulated in the ER and induce ER stress signals

Using double-labeled immunofluorescence staining, we observed consistent colocalization of pre-S mutant proteins and the ER protein calregulin, suggesting that these pre-S mutant proteins, both the Δ S1-LHBs and Δ S2-LHBs, accumulate in the ER. Ultrastructurally, Δ S2-LHBs could induce a significant proliferation of the ER to an extent that is similar to the characteristics of GGH in the liver (Fig. 1d). The accumulation of pre-S mutants in ER may subsequently induce ER stress signals, leading to the induction of ER chaperones (GRP78 and GRP94).^(23,26) ER stress, also called the UPR in mammalian cells, is a cellular defense mechanism that responds to unfolded viral proteins or perturbed ER functions. Expression of viral gene products usually induces the UPR, which is sensed by two ER transmembrane kinases (IRE1 and PERK), and one ER transmembrane transcription factor (ATF-6).⁽²⁷⁾ These three UPR sensors are associated with ER chaperone GRP78/BiP when resting, and are dissociated from GRP78 upon ER stress. In our laboratory, we have demonstrated that the GRP78 gene is induced significantly in both types of GGH and in HCC, compared with non-HBs-expressing hepatocytes.^(23,28) It has been reported that the induction of GRP78 may prevent cells from death by forming a complex with caspase-7 and caspase-12, and ER stress-regulated translation may increase tolerance to extreme hypoxia and promote tumor growth.^(29,30) The activation of ER-stress downstream molecules such as ATF-6, GRP78 and XBP-1 is believed to be involved in hepatocarcinogenesis.⁽³¹⁾ These data suggest that ER stress may play a contributing role in cell transformation, especially in HCC.

Pre-S mutant-induced ER stress may induce genomic instability through oxidative stress, increased DNA damage and mutation frequency

The pre-S mutants may induce oxidative DNA damage through ER stress signaling pathways. HuH-7 cells carrying the pre-S mutant LHBs exhibited enhanced levels of reactive oxygen species and oxidative DNA damage.⁽³²⁾ Furthermore, pre-S mutant LHBs can induce mutations on the X-linked *hprt* gene. Oxidative DNA damage could also be observed in livers of transgenic mice carrying pre-S mutant LHBs, as well as in GGH.⁽³²⁾ Therefore, through ER stress signaling pathways, the pre-S mutant LHBs can induce oxidative stress and lead to oxidative DNA damage of HBV-infected hepatocytes. The oxidative DNA damage caused by pre-S mutant LHBs may result in genomic instability and mutation of liver cells, and ultimately lead to HCC.

Recently, preliminary studies from our group revealed that Δ S2-LHBs, but not wild-type or Δ S1-LHBs, could induce hyperphosphorylation of tumor suppressor RB. The Δ S2-LHBs could interact directly with JAB1, dissociating JAB1 from the JAB1-IRE1 complex in the ER lumen and causing JAB1 to translocate to cell nuclei. JAB1 is an important factor for modulating the level of cyclin-dependent kinase inhibitor p27^{Kip1}, and targets nuclear p27^{Kip1} to the cytosolic 26S proteasome for degradation. The degradation of p27^{Kip1} activates

the phosphorylation of RB. Therefore, the combined events of oxidative DNA damage and RB hyperphosphorylation may represent two independent events for hepatocellular carcinogenesis associated with the ER viral protein Δ S2-LHBs.

Pre-S mutants may upregulate COX-2 through NF- κ B and p38 MAPK

Overexpression of COX-2 has been detected in many types of cancer and is linked to disease progression and survival of candidate cells. One of the many roles of pre-S mutant LHBs is to regulate the expression of COX-2.⁽³³⁾ Constitutive or inducible expression of pre-S mutant LHBs induces the expression of COX-2 and prostaglandin E2 in immortalized mouse liver ML-1 cells and in transformed human HuH-7 cells. Transgenic mice expressing pre-S mutant LHBs express higher levels of COX-2 protein in liver and kidney tissues. Similarly, increased expression of COX-2 mRNA was observed in human HCC tissues expressing pre-S mutant LHBs. COX-2 induction is apparently important for the anchorage-independent growth conferred by the expression of pre-S mutant LHBs. Addition of etodolac, a specific COX-2 inhibitor, can abolish the growth of ML-1 cells expressing pre-S mutant LHBs in soft agar.

The induction of COX-2 is mediated mainly through transcriptional activation as actinomycin D (an inhibitor of RNA transcription) can attenuate the expression of COX-2 significantly. The transcription factor NF- κ B is essential for transactivation of the COX-2 promoter.⁽³⁴⁾ Nuclear translocation of NF- κ B, especially the p65 subunit, is observed when cells are treated with tunicamycin and brefeldin A, two ER stress inducers. NF- κ B inhibitor can also block the induction of COX-2 by either an artificial ER stress inducer such as tunicamycin or the expression of pre-S mutant LHBs. Similar to our findings, NF- κ B has been proposed to play a major role in the progression of inflammation-associated cancer.^(35,36) The activation of NF- κ B is not only involved in the traditional degradation of I κ B but is also regulated by p38 MAPK. Inhibition of p38 MAPK does not affect the nuclear translocation of NF- κ B but does inhibit NF- κ B DNA binding activity⁽³³⁾ and attenuate the induction of COX-2.

Taken together, the above-described role of NF- κ B and COX-2 in the signal transduction of pre-S mutant LHBs suggests that they may act as potential preventive or therapeutic targets for HBV-related HCC. COX-2 inhibitor has been verified for the chemoprevention of many human cancers.^(37,38) The chemoprevention of liver cancer with COX-2 inhibitor may deserve future clinical trials.

Pre-S2 mutant Δ S2-LHBs can selectively upregulate cyclin A and induce cell cycle progression

Cyclin A is involved in both DNA synthesis and centrosome duplication during the cell cycle.⁽³⁹⁾ It has been reported that both cyclin A and cyclin E are enhanced in HCC tissues and may be associated with tumor invasiveness and metastasis.^(40,41) Using cDNA microarray analysis, we observed that cyclin A, along with other cell cycle-regulated genes, was induced by Δ S2-LHBs.⁽²⁸⁾ The induction of cyclin A was shown to be initiated via the specific transactivator function of Δ S2-LHBs,

independent of ER stress signals. The expression of Δ S2-LHBs in hepatocytes led to cell cycle progression under strong ER stress conditions and enhanced 5-bromo-2-deoxyuridine (BrdU)-incorporation with a multinucleation phenotype. Histopathological examinations revealed that cyclin A expression was enhanced in GGH, HCC tissues and transgenic mouse livers.⁽²⁸⁾ One interesting finding is the tremendous expression of cyclin A in the cytoplasm of hepatocytes induced by Δ S2-LHBs. Although cyclin A functions predominantly in the nucleus, cytoplasmic expression of cyclin A may contribute to centrosome duplication.^(39,42) It is therefore interesting to clarify whether alteration of the subcellular localization of cyclin A could affect centrosome duplication that may subsequently contribute to cell aneuploidy.⁽⁴³⁾ Recently, our study suggested that cytoplasmic localization of cyclin A is associated with its N-terminal truncation by a calcium-dependent protease, which is activated by ER stress (H. C. Wang and I. J. Su, unpublished data). These data suggest that cytoplasmic cyclin A may be initiated by ER stress and may contribute to aberrant centrosome duplication. The increased multinucleation and DNA aneuploidy observed in Δ S2-LHBs transgenic mouse livers supports the potential role of ER stress and impaired cyclin A during the development of HCC.

Pre-S mutant LHBs can induce dysplastic changes and tumor formation in transgenic mice

Hepatitis B virus transgenic mice have been produced both with complete HBV genomes that can support viral replication and with selected viral genes, including three surface proteins (large, middle and small HBs), HBx, core and precore proteins.⁽⁴⁴⁾ Transgenic mouse studies have revealed a contributing role for HBx in hepatocarcinogenesis but the importance of HBs remains debatable.^(45,46) The overexpression of LHBs protein in transgenic mice has been shown to be cytopathic, and could lead to liver injury, regenerative hyperplasia, chronic inflammation, oxidative DNA damage, hepatocyte aneuploidy and eventually progression to HCC.^(47,48) In our laboratory, we demonstrated that Δ S2-LHBs can induce nodular proliferation and dysplasia of hepatocytes in transgenic mice,⁽²⁸⁾ and tumor development was demonstrated recently (Fig. 2).

Presence of pre-S mutants carries a high risk of developing HCC and represents a potential prophylactic target to prevent HCC development

To test whether patients with HBV pre-S mutants carry a higher risk of HCC development, a case-control study nested in a cohort of 4155 HBsAg-seropositive residents was conducted to assess the risk of developing HCC in HBV carriers with or without pre-S mutants at enrolment. This nested case-control analysis involved 68 men with newly diagnosed HCC and 132 matched controls. Among these cases, 14 men with HCC (20.6%) and 11 controls (8.3%) had detectable pre-S mutants in serum samples at enrolment. After adjustment for age and HBeAg serostatus, the patients harboring pre-S mutants at enrolment had a higher risk of developing HCC than those

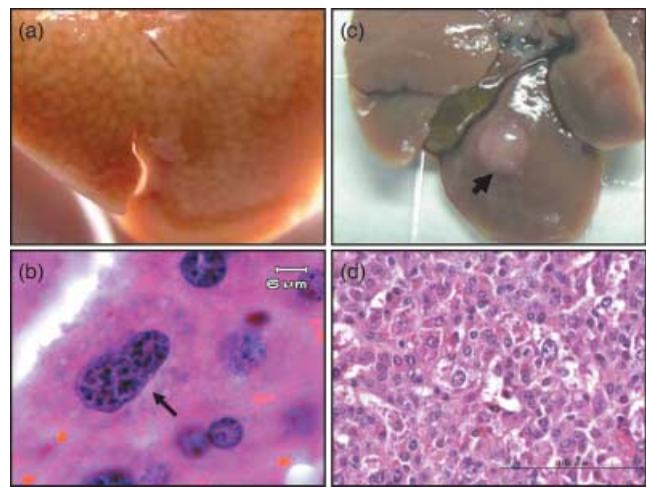


Fig. 2. Large surface protein with a deletion at the pre-S2 region (Δ S2-LHBs) induces nodular proliferation, nuclear dysplastic changes and hepatocellular carcinoma on transgenic mice. (a) A transgenic mouse liver revealed nodular proliferation. (b) Nuclear dysplastic changes on transgenic liver. Black arrow indicates one affected hepatocyte with enlarged nucleus, hyperchromatism and nuclear pleomorphism. (c) Tumor formation on Δ S2-LHBs transgenic mice. (d) Pathological finding of hepatocellular carcinoma on Δ S2-LHBs transgenic mice.

without pre-S mutants during the 10-year follow-up period (OR = 3.2, 95% CI = 1.3–7.9, $P = 0.01$) (H. I. Yang and C. J. Chen, unpublished data). These data indicate that the emergence of pre-S mutants may carry a high risk of developing HCC in patients with chronic HBV infection.

HBV pre-S mutants may provide a model for viral carcinogenesis through latent ER stress signals

The potential role of HBV pre-S mutants in the pathogenesis of HCC provides us with a model for viral carcinogenesis. The viral proteins may adopt a complex mechanism to escape from the host immune response by manipulating the machinery of cytoplasmic processing through the proteasome ubiquitination system. In this process, transport from the ER to the Golgi apparatus and cell membrane is manipulated meticulously by many viruses to escape from immune attack. In chronic HBV infection, a series of mutations under evolutionary cytotoxic T lymphocyte (CTL) immune pressure may result in the emergence of pre-S mutants that accumulate in the ER.^(21,23,26,49,50) Retention of viral mutant proteins in the ER may induce ER stress and lead to apoptosis or, alternatively, the cells may try to survive through the activation of cell proliferation-related signals such as COX-2 and cyclin A to initiate cell proliferation (Fig. 3).^(28,32,33) In the presence of DNA damage and genomic instability, the proliferating cells may progress to tumor formation.

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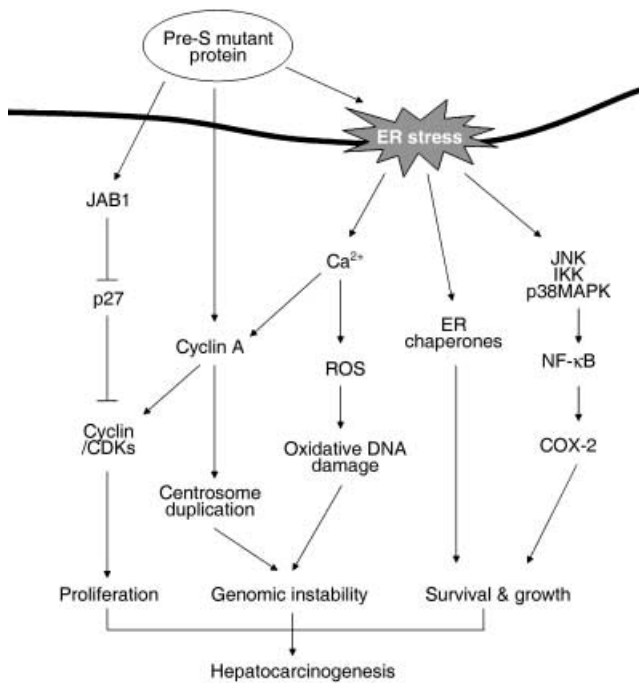


Fig. 3. Oncogenic pathway of hepatitis B virus pre-S mutant proteins. CDK, cyclin dependent kinase; COX-2, cyclooxygenase-2; ER, endoplasmic reticulum; IKK, IκB kinase; JAB1, Jun activation domain-binding protein 1; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor κB; ROS, reactive oxygen species.

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