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Hepatitis B Virus Core Promoter Mutations Contribute to Hepatocarcinogenesis by Deregulating SKP2 and its Target, p21

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Abstract

Background & Aims—Clinical studies have associated hepatitis B virus (HBV) core promoter (CP) mutations with an increased risk of hepatocellular carcinoma (HCC). The CP region overlaps with the *HBV* × (*HBx*) gene, which has been implicated in hepatocarcinogenesis. The cyclin kinase inhibitor p21WAF1/CIP1 is an important regulator of cell cycle progression and proliferation. We determined whether HBx mutants that result from mutations in the CP deregulate p21 and these processes.

Methods—We constructed a series of HBx mutants with changes in the CP region that correspond to A1762T/G1764A (TA), T1753A, T1768A, or a combination of these (combo) and expressed them, along with wild-type HBx under control of its endogenous promoter, in primary human hepatocytes (PHH) and HepG2 cells. We then analyzed the effects of CP mutations on expression and degradation of p21 and the effects on cell cycle progression and proliferation.

Results—The combo mutant decreased levels of p21 and increased cyclin E expression in PHH and HepG2 cells. The combo mutant, but not HBx with single or double CP mutations, accelerated p21 degradation in HepG2 cells. The combo mutant increased expression of S-phase kinase-associated protein 2 (SKP2) in PHH and Huh7 cells. Silencing of SKP2 abrogated the effects of CP mutations on p21 expression. The kinetics of p21 expression correlated with changes in cell cycle distribution. The combo mutant accelerated cell cycle progression; p21 overexpression restored G1 arrest.

Conclusions—HBx mutants with changes that correspond to a combination of CP mutations upregulate SKP2, which then down-regulates p21 via ubiquitin-mediated proteasomal degradation. CP mutations might increase the risk for HCC via this pathway.

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Liver cancer; genetics; virology; tumorigenesis

People with chronic hepatitis B virus (HBV) infection have approximately a 100-fold increased risk of developing hepatocellular carcinoma (HCC) compared to those without HBV infection.¹ Among those with chronic HBV infection, several viral factors have been reported to be associated with an increased risk of HCC: HBV genotype C, Aa and F, core promoter mutations, and high viral load.²⁻⁴

Clinical studies have shown that the common double mutation A1762T/G1764A (TA) in the HBV basal core promoter (CP) region is independently associated with HCC. In a study of 2762 persons with chronic HBV infection, the TA core promoter mutation was associated with a hazard ratio of 1.73 for developing HCC.³ Besides the TA mutation, other core promoter mutations, notably T1753V and T1768A, have also been reported to be associated with an increased risk of HCC.^{5, 6} These other mutations were almost invariably found in association with the TA mutation, suggesting a key role for the TA double mutation in hepatocarcinogenesis. Longitudinal studies have shown that the TA mutation is selected earlier – many years before HCC diagnosis — while the other core promoter mutations emerge later, closer to the time of HCC diagnosis.^{5, 7, 8} Cross-sectional studies have found that while the TA mutations were more frequently detected in patients with HCC, they can also be detected in a high percentage of patients without HCC.^{6, 9} These clinical data suggest that TA mutations are important in the early stages of carcinogenesis but that additional mutations may be necessary for HCC development.

The mechanisms by which core promoter mutations induce hepatocarcinogenesis are not clear. *In vitro* studies suggest that core promoter mutations enhance HBV replication;^{10, 11} however, clinical studies have found that the presence of the TA mutation is not always associated with higher serum HBV DNA levels.¹²⁻¹⁴

The HBV $\times$ gene encodes a 17-KDa protein called HBx. HBx protein is a multifunctional regulator that modulates transcription, protein degradation, signal transduction, cell cycle control and apoptosis.^{15, 16} HBx protein has been incriminated in hepatocarcinogenesis. The core promoter region overlaps with the coding sequence for the C terminus of HBx, where the function of transactivation resides.¹⁷ Mutations in the core promoter region alter the coding sequence of the overlapping $\times$ gene and may alter the biological functions of the HBx protein thereby increasing the risk of HCC.¹⁸

Deregulation of cell cycle related protein has been suggested to be an important factor in HCC development and progression.¹⁹ Cell cycle progression is regulated by cyclin-dependent kinases and their specific inhibitors.²⁰ p21^{WAF1/CIP1} (p21) is a universal inhibitor of cyclin-dependent kinases.²¹ It halts cell cycle progression in response to various stimuli.²² p21 deficiency may increase carcinogenesis in combination with other oncogenic mutations.²³⁻²⁶ A few studies have linked HBx to alterations in p21 expression but the results are conflicting.²⁷⁻²⁹ These discrepancies may be related to differences in HBx gene sequence or expression level as well as in the cell lines used.

Increasing evidence indicates that targeted proteolysis of cell cycle related proteins by the ubiquitin-proteasome system plays a central role in regulating cellular growth.³⁰ The S-phase kinase-associated protein 1 (SKP1)/CUL1/F-box (SCF) complex is an ubiquitin E3 ligase responsible for recognizing and recruiting target proteins for polyubiquitylation.³⁰ The SCF complex has been shown to regulate G1-S transition by posttranscriptional regulation of tumor suppressors and may play an important role in promoting or preventing

cellular transformation and proliferation.^{30, 31} One of the F-box proteins in the SCF complex--the S-phase kinase-associated protein 2 (SCF^{SKP2}) has been shown to be involved in ubiquitination of p21.³² We hypothesized that HBx modulates p21 expression through SKP2 and the ubiquitin-proteasome system. In this study, we produced WT and mutant HBx (with mutations corresponding to changes in the core promoter region that have been reported to be associated with HCC in clinical studies) that are expressed under the control of its endogenous enhancer and promoter in both human primary hepatocytes and hepatoma cell lines to compare the effect of mutant versus WT HBx on p21 expression and the pathways that modulate p21 expression.

Materials and Methods

Plasmid construction

Luciferase reporter plasmids driven by various lengths of the 5'-flanking region of HBx: (-69), (-109), (-139), (-314), and (-474) were constructed from plasmids carrying a 1.5-mer HBV genome of genotypes A, B and C. Expression plasmids encoding HBx from HBV genotypes A, B, and C were generated by amplifying the open reading frame and enhancer/promoter region (nt1060~1838) of the $\times$ gene from the same HBV 1.5-mers (primer sets used are shown in Supplementary Table 1). A FLAG epitope was introduced at the C-terminus of HBx. The amplified fragment was cloned into the pGL3-Basic vector (Promega, Madison, WI) which lacks promoter and enhancer sequences, with excision of the luciferase gene. The HBx-Flag constructs were verified by direct sequencing. HBx protein expression was determined by anti-FLAG antibody (Stratagene, La Jolla, CA).

Site-directed mutagenesis

To generate HBx mutants that correspond to mutations in the basal core promoter region, site-directed mutagenesis was performed at amino acid positions I127N (T1753A), K130M/V131I (A1762T/G1764A) (abbreviated as TA mutant) and F132Y (T1768A) using a Quikchange kit (Stratagene) according to the manufacturer's protocol. In addition, a combination mutant of I127N/K130M/V131I/F132Y (abbreviated as Combo mutant) was generated. All mutants were verified by DNA sequencing.

Primary human hepatocyte culture and transduction

Primary human hepatocytes (PHH) were purchased from Clonetics (Lonza, Walkersville, MD). The $\times$ gene and its upstream sequence (nt 1060–1838) was inserted between XbaI and NotI sites of the lentivirus expression vector pLentiLox 3.7 which was kindly provided by Dr. Thomas Lanigan. The resultant pLentiLox3.7-HBx constructs were verified by direct sequencing. To generate lentivirus, plasmids pLentiLox 3.7-based HBx, psPAX2 and pMD2.G were cotransfected into 293T cells using FuGENE (Roche). For transduction of PHH, cells were incubated for 6h with concentrated lentivirus preparations with 8 μ g/ml polybrene (Sigma) on day 3. Culture medium was then changed and cells were maintained for 48h. Western blot was performed to determine p21, SKP2, and cyclin E expression (details in Supplementary materials).

Cell cycle analysis

DNA content was assessed by staining ethanol-fixed cells with propidium iodide and measured by FACScalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ). The percentage of cells in the G0/G1, S, and G2/M phases of the cell cycle was analyzed using ModFIT software (Verity Software House, Topsham, ME) (details in Supplementary materials).

5-Bromo-2'-deoxyuridine incorporation assay

5-Bromo-2'-deoxyuridine (BrdU) incorporation assay was used to analyze DNA synthesis of HBx expressing hepatoma cells (details in Supplementary materials).

p21 and SKP2 expression in human livers

Western blotting and immunohistochemistry were performed on explant livers from 14 patients with chronic HBV infection to compare p21 and SKP2 expression in patients with WT versus TA core promoter mutation (Supplementary material).

Statistical analysis

Statistical analysis was performed using GraphPad Prism4 software. The unpaired, two-tailed *t* test was used for the comparison of parameters between groups. Values of *P* < .05 were considered significant. Data throughout this manuscript are shown as mean ± SD.

Results

Construction of HBx expression plasmids under endogenous promoter/enhancers

HBx promoter luciferase reporter plasmids containing various lengths of the HBx 5'-flanking region: (−69/+1), (−109/+1), (−139/+1), (−314/+1), and (−474/+1) of genotypes A, B and C were transiently transfected into HepG2 cells to determine their promoter activity. Results indicated that constructs (−314/+1) and (−474/+1) exhibited the highest luciferase activity while constructs (−109/+1) and (−139/+1) displayed a modest promoter activity as compared to the pGL3-Basic vector (Figure 1A). The same results were obtained across genotypes A, B and C, and were also confirmed in Huh7 and NIH 3T3 cells (data not shown). Plasmids encoding WT and mutant HBx for genotypes A, B, and C driven by the HBx enhancer 1 and HBx promoter (−314 to +465) were constructed. We also constructed plasmids encoding mutant HBx (K130M, V131I) proteins of all three genotypes, corresponding to the core promoter TA mutation. HBx protein expression in HepG2 cells transfected with WT and TA mutant HBx of genotypes A, B and C was similar for all of the HBx constructs regardless of genotype or WT versus TA mutant status (Figure 1B).

Combo mutant downregulates p21 expression

We first investigated p21 expression in hepatoma cell lines expressing WT and mutant HBx. Results showed that TA mutant HBx had no effect on p21 expression in HepG2 cells compared with a negative control plasmid, while WT HBx increased p21 expression. This phenomenon was observed for HBx from HBV genotypes A, B, as well as C (Figure 2A). Because the effect of WT and TA mutant HBx expression on p21 levels was independent of HBV genotype, subsequent experiments were performed using genotype C HBx plasmid. In addition to the TA mutant, we studied three HBx mutants in the genotype C background that corresponded to the following core promoter mutations: (1) T1753A, (2) T1768A, (3) combination of T1753A, TA, and T1768A (Combo mutant). Combo mutant significantly decreased p21 expression while single or double CP mutations had minimal effect on p21 expression (Figure 2B).

We next asked whether the effect of HBx mutant on p21 expression seen in human hepatoma cell lines is also observed in PHH. Similar to our findings in HepG2 cells, p21 expression was markedly decreased by Combo mutant, unchanged by TA mutant, and mildly increased by WT HBx (Figure 2C).

Finally, we examined p21 expression by immunoblotting and immunohistochemistry in liver tissues from patients with WT and TA mutant chronic HBV infection. p21 expression was

lower in liver tissues from patients with TA mutant compared to those with WT HBV (Supplementary Figure 1).

Combo mutant stimulates S-phase progression

To determine if HBx-induced changes in p21 expression affect cell cycle progression, flow cytometry was performed 72 hours post-transfection. Consistent with the effect of Combo mutant on decreasing p21 expression, we found that Combo mutant significantly increased the proportion of cells in S phase ($30.8 \pm 3.1\%$ for Combo mutant vs $14.1 \pm 1.9\%$ and $7.0 \pm 0.6\%$ for control plasmid and WT HBx, respectively, $P < .01$ and $P < .001$, Figure 3A).

Transfection with TA mutant and other single HBx mutant did not impact cell cycle progression (Supplementary Figure 2A and 2B). Similar results were obtained for genotypes A and B and for Huh7 cells (data not shown).

To determine whether cell cycle changes observed were truly related to p21 expression, we forced p21 overexpression and found a significant decrease in the proportion of cells in S phase cotransfected with Combo mutant and p21 compared to cells transfected with Combo mutant only ($13.3 \pm 0.5\%$ vs $32.0 \pm 5.3\%$, $P < .01$, Figure 3B). These data implied that restoration of p21 expression in Combo mutant HBx expressing cells can reduce the progression of cells into S phase. Also, a time course study of p21 expression and cell cycle distribution showed that the increase in p21 expression induced by WT HBx was paralleled by a corresponding decrease in the proportion of cells in S phase (Figure 3C). Finally, knocking down p21 level by RNA interference increased the proportion of WT HBx-expressing cells in S phase compared to cells transfected with a nontargeting siRNA ($24.0 \pm 5.5\%$ vs $9.4 \pm 1.1\%$, $P < .01$, Figure 3D). These results clearly indicate that the effect of HBx on cell cycle distribution is mediated principally through p21.

To further verify the increase in S-phase population in Combo mutant expressing cells is due to an increase in *de novo* DNA synthesis, BrdU incorporation assay was performed in synchronized HepG2 cells. The BrdU incorporation index of Combo mutant expressing cells was significantly higher than that of TA and WT HBx ($P < .05$ and $P < .01$, respectively, Figure 3E), indicating that Combo mutant stimulated S-phase entry. S-phase entry was also increased in unsynchronized Huh7 cells transfected with Combo mutant (data not shown). Stimulation of S-phase was attenuated by the re-introduction of p21 (Figure 3E).

To determine whether Combo mutant influences S phase progression in PHH, we monitored expression of cyclin E—a key regulator of G1/S phase transition. Cyclin E protein level was higher in Combo mutant expressing PHH than TA and WT HBx expressing PHH (Figure 3F). Similar result was observed in HepG2 cells (data not shown).

Combo mutant increases cellular proliferation and colony formation

To examine whether the altered expression of p21 by core promoter mutations affect cellular proliferation, the growth rates in HepG2 cells stably expressing WT, TA and Combo mutant HBx were compared. As expected from their effects on p21 expression, Combo mutant HBx expressing cells grew significantly more quickly than WT ($P < .001$) and TA mutant ($P < .036$) HBx expressing cells (Figure 4A). Similar results were seen in Huh7 cells (data not shown).

To study the transformation potential of core promoter HBx mutants, anchorage-dependent growth was analyzed by soft agar assay in NIH3T3 cells stably transfected with mutant or WT HBx. The results showed that a mean of $6.3 \pm 0.3\%$ and $1.73 \pm 0.29\%$ cells expressing Combo and TA HBx mutant, respectively, formed colonies compared to $0.63 \pm 0.14\%$ cells expressing WT HBx ($P < .01$ and $P < .031$, respectively) and $1.21 \pm 0.31\%$ cells stably transfected with an empty vector ($P < .05$ and $P = .261$, respectively, Figure 4B).

Combo mutant accelerates p21 degradation

To further investigate the mechanisms by which HBx regulates p21 expression, the effect of HBx mutants on p21 protein stability/degradation and p21 transcription was studied. First, a protein stability experiment in cycloheximide-treated HepG2 cells showed that p21 stability was decreased in Combo mutant transfected cells, unchanged in TA mutant HBx-transfected cells, and increased in WT HBx-transfected cells compared to empty vector-transfected cells (Figure 5A). Similar results were also observed in Huh7 cells (data not shown). These data indicate that combination of CP mutations accelerated p21 protein degradation accounting for the observed decrease in steady-state p21 levels. To unravel the mechanism by which HBx mutant regulates p21 stability, cells were treated with a proteasome inhibitor MG132. The effects of mutant HBx on p21 stability were abolished (Figure 5B) suggesting that HBx regulation of p21 stability occurs via the ubiquitin-proteasomal system.

Next, we used a p21-luciferase reporter plasmid that encodes the luciferase gene under the control of a 2.4 kb p21 promoter to determine whether CP mutations have an effect on the transcription of p21. The luciferase reporter assay indicated that Combo mutant HBx downregulated p21 transcription (Figure 5C). Because p21 transcription is positively regulated by p53, the role of p53 on HBx-regulated p21 expression was studied. We found that Combo mutant HBx decreased steady-state p21 expression regardless of p53 silencing (Figure 5D). Furthermore, forced p53 overexpression had no effect on p21 protein level in Combo mutant HBx transfected Hep3B cells, which are p53-null (data not shown).

Core promoter mutations regulate p21 expression in a SKP2-dependent manner

Given that SKP2, a component of the SCF ubiquitin E3 ligase, can target p21 for proteasomal degradation, we further determined if the effect of HBx on p21 degradation is dependent on SKP2. Data from both Huh7 cells (Figure 6A) and PHH (Figure 6B) demonstrated that compared to control, SKP2 expression increased in Combo mutant and remained unchanged in TA HBx expressing cells. SKP2 expression was significantly decreased in Huh7 cells (Figure 6A) and slightly decreased in PHH (Figure 6B) transfected with WT HBx. Similar findings were observed in liver tissues from patients with chronic HBV infection (Supplementary Figure 1).

To further delineate the role of SKP2 in regulating p21 expression, we knocked down SKP2 by RNA interference and determined the effect on p21 protein expression and cell cycle distribution. Silencing of SKP2 resulted in an increase of p21 expression in cells transfected with TA and Combo mutant HBx, such that the steady-state p21 level was similar to WT HBx-transfected cells (Figure 6C). Furthermore, SKP2 silencing resulted in similar cell cycle distributions regardless of which HBx protein (WT, TA, or Combo) was expressed (Figure 6C). SKP2 knock-down also abolished the enhancement of S phase entry by Combo mutant HBx (Figure 6D). These results indicate that HBx regulates p21 expression primarily through ubiquitination, which in turn requires the SKP2-containing SCF E3 ligase.

Discussion

Clinical observations have suggested that HBV core promoter mutations generally accumulate in a stepwise fashion prior to the development of HCC, often beginning with the TA double mutation. Because mutations in the core promoter region found in patients with HCC alter the coding sequence of the overlapping HBx gene and HBx protein has been previously incriminated in hepatocarcinogenesis, we investigated the impact of core promoter mutations on the biological function of HBx protein.

Our results in HepG2 and Huh7 cells indicate that Combo mutant HBx I127N/K130M/V131/I/F132Y with changes corresponding to core promoter mutations T1753A/A1762T/G1764A/

T1768A that are typically found in patients close to the time of diagnosis of HCC decreased p21 expression while TA double mutation or other single CP mutants had a more modest effect on p21 downregulation compared to control. To determine whether these findings could be replicated under more physiological conditions, we tested the effect of CP mutations on p21 expression in primary human hepatocytes. Similar to our findings in hepatoma cell lines, Combo mutant HBx decreased p21 protein level >3-fold in PHH, while TA mutant and WT HBx had minimal effect on p21 expression.

Cell cycle analysis and BrdU incorporation assay showed that Combo mutant HBx increased the proportion of cells in S phase and extent of cellular DNA synthesis. Forced overexpression of p21 restored G1 arrest and attenuated G1/S-phase transition in Combo mutant expressing cells, indicating that the role of Combo mutant on cell cycle progression is mediated principally through p21. Combo mutant HBx was also shown to increase cellular proliferation and anchorage-independent growth. We also demonstrated that Combo mutant upregulated cyclin E expression in PHH. Cyclin E can bind to CDK2 and the cyclin E/CDK2 complex can promote G1/S-phase transition and act as a switch for various cellular processes including initiation of DNA replication³³. We showed that Combo mutant HBx decreases p21 (which inhibits S-phase entry) and increases cyclin E (which enhances S-phase entry) in PHH, providing indirect evidence to support that Combo mutant may contribute to S-phase entry, DNA replication and cellular proliferation.

p21 exerts two known biological activities: promoting cell cycle arrest and cellular senescence in response to various stimuli.³⁴ Our results here suggest that loss of p21 in Combo mutant HBx expressing hepatocytes deregulates cell cycle control and promotes anchorage-independent growth. While p21 deficiency in isolation may not be sufficient to increase tumor formation, several studies have shown that p21 deficiency can cooperate with other oncogenic mutations to enhance carcinogenesis.^{23–26} Also, recent studies for the role of p21 in HCC have indicated p21 as a key downstream regulator of some novel HCC related proteins, an effector in strong antitumor response against HCC and a target of some anticancer agents.^{35–37} These data are consistent with a role of p21 in multi-step tumorigenesis. We speculate that the decreased p21 due to accumulation of multiple CP mutations during the course of chronic HBV infection may be accompanied by the accumulation of other oncogenic mutations further increasing the risk of HCC.

To determine how CP mutations regulate p21 expression, we examined the effects of WT and mutant HBx on p21 transcription as well as p21 protein stability. Our studies showed that Combo mutant HBx decreased p21 protein half-life and this effect was mediated through the ubiquitin-proteasomal system. Levels of SKP2, the S-phase kinase-associated protein 2, were highest in Combo mutant, intermediate in TA, and lowest in WT HBx expressing PHH and Huh7 cells. Our finding that SKP2 plays a key role in HBx regulation of p21 is novel but not surprising. Elevated SKP2 expression has been observed in many human cancers, including HCC.^{38, 39} In addition to p21, the SCF E3 ubiquitin ligase complex containing SKP2 also target other cell cycle regulators such as p27, p57, and p130 for polyubiquitination.³⁰ Although we did not specifically examine the expression of these other factors in response to HBx expression, we hypothesize that the ability of mutant HBx to promote hepatocarcinogenesis may be mediated by deregulation of additional factors besides p21. SKP2 is a common downstream effector of multiple mitogenic cascades, including Ha-Ras, AKT, JAK/STAT, and Wnt/beta-catenin, which are implicated in human hepatocarcinogenesis,^{19,39} and SKP2 upregulation may favor cellular transformation and tumor progression.³⁰ Therefore, SKP2 may be a therapeutic target for HBV-related HCC.

Compared to previous studies which have analyzed the impact of HBx on p21 expression^{27–29}, ours are significant for the following reasons. First, prior studies did not

provide details on HBx protein sequence and it is unclear whether only WT HBx was studied. Our study showed that HBx plasmids with mutations corresponding to the core promoter mutations found in association with HCC in the clinic had opposite effects on p21 expression compared to WT HBx. Second, many in vitro studies used exogenous promoters which may have resulted in nonphysiological expression levels of HBx. In this study, we used the endogenous HBx enhancer/promoter to drive HBx expression. Third, we showed for the first time that HBx regulates p21 via SKP2. Finally, we demonstrated that combination of CP mutations not only down regulated p21 expression but also upregulated SKP2 level in primary human hepatocytes as well as in cancer cell lines.

This study has several potential limitations. First, HBx alone was studied. It is possible that the effects of core promoter mutations on p21 expression and cell cycle progression may be different when HBx is expressed in the context of the entire HBV genome. Second, HBx might also affect other cell cycle regulators such as p27 and p57. Ongoing studies are investigating the interaction of HBx with other cell cycle regulators.

In summary, our study provides a possible mechanism by which HBV core promoter mutations are associated with an increased risk of HCC. The results suggest that core promoter mutations seen in patients with HCC promote carcinogenesis possibly via downregulation of p21 expression and in turn acceleration of cell cycle progression, and enhancement of colony formation and cellular proliferation. These effects are more pronounced when a combination of core promoter mutations typically seen at the time of HCC diagnosis is present. The marked difference between the effect of Combo mutant vs TA mutant on p21 and SKP2 expression in primary human hepatocytes may explain why a combination of CP mutations has a stronger association with HBV-related HCC than TA mutant alone. Deregulation of p21 expression appears to occur mainly at the posttranslational level through SKP2-dependent ubiquitin-proteasomal pathways. We acknowledge that core promoter mutations may also promote hepatocarcinogenesis through other mechanisms; elucidating these pathways will provide further insight into the oncogenic role of core promoter mutations and may lead to the development of novel therapeutic strategies to prevent HBV-related HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

HCC	hepatocellular carcinoma
HBx	hepatitis B virus X-protein
TA mutant	A1762T/G1764A mutant
Combo mutant	T1753A/A1762T/G1764A/T1768A mutant

WT	wild type
SKP	S-phase kinase-associated protein
MG132	carbobenzoxy-L-leucyl-leucyl-leucinal
SCF	SKP1/CUL1/F-box ubiquitin ligase complex
PHH	primary human hepatocytes
BrdU	5-bromo-2'-deoxyuridine

References

1. Yang HI, Lu SN, Liaw YF, et al. Hepatitis B e antigen and the risk of hepatocellular carcinoma. *N Engl J Med*. 2002; 347:168–174. [PubMed: 12124405]
2. Yuen MF, Tanaka Y, Shinkai N, et al. Risk for hepatocellular carcinoma with respect to hepatitis B virus genotypes B/C, specific mutations of enhancer II/core promoter/precore regions and HBV DNA levels. *Gut*. 2008; 57:98–102. [PubMed: 17483190]
3. Yang HI, Yeh SH, Chen PJ, et al. Associations between hepatitis B virus genotype and mutants and the risk of hepatocellular carcinoma. *J Natl Cancer Inst*. 2008; 100:1134–1143. [PubMed: 18695135]
4. Chen CJ, Yang HI, Su J, et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA*. 2006; 295:65–73. [PubMed: 16391218]
5. Liu S, Zhang H, Gu C, et al. Associations between hepatitis B virus mutations and the risk of hepatocellular carcinoma: a meta-analysis. *J Natl Cancer Inst*. 2009; 101:1066–1082. [PubMed: 19574418]
6. Guo X, Jin Y, Qian G, et al. Sequential accumulation of the mutations in core promoter of hepatitis B virus is associated with the development of hepatocellular carcinoma in Qidong, China. *J Hepatol*. 2008; 49:718–25. [PubMed: 18801591]
7. Chou YC, Yu MW, Wu CF, et al. Temporal relationship between hepatitis B virus enhancer II/basal core promoter sequence variation and risk of hepatocellular carcinoma. *Gut*. 2008; 57:91–97. [PubMed: 17502344]
8. Kuang SY, Jackson PE, Wang JB, et al. Specific mutations of hepatitis B virus in plasma predict liver cancer development. *Proc Natl Acad Sci U S A*. 2004; 101:3575–3580. [PubMed: 14990795]
9. Ito K, Tanaka Y, Kato M, et al. Comparison of complete sequences of hepatitis B virus genotype C between inactive carriers and hepatocellular carcinoma patients before and after seroconversion. *J Gastroenterol*. 2007; 42:837–844. [PubMed: 17940837]
10. Parekh S, Zoulim F, Ahn SH, et al. Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants. *J Virol*. 2003; 77:6601–6612. [PubMed: 12767980]
11. Buckwold VE, Xu Z, Chen M, et al. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. *J Virol*. 1996; 70:5845–5851. [PubMed: 8709203]
12. Chu CJ, Keeffe EB, Han SH, et al. Prevalence of HBV precore/core promoter variants in the United States. *Hepatology*. 2003; 38:619–628. [PubMed: 12939588]
13. Fang ZL, Sabin CA, Dong BQ, et al. The association of HBV core promoter double mutations (A1762T and G1764A) with viral load differs between HBeAg positive and anti-HBe positive individuals: a longitudinal analysis. *J Hepatol*. 2009; 50:273–280. [PubMed: 19070921]
14. Chun YK, Kim JY, Woo HJ, et al. No significant correlation exists between core promoter mutations, viral replication, and liver damage in chronic hepatitis B infection. *Hepatology*. 2000; 32:1154–1162. [PubMed: 11050069]
15. Bouchard MJ, Schneider RJ. The enigmatic $\times$ gene of hepatitis B virus. *J Virol*. 2004; 78:12725–12734. [PubMed: 15542625]

16. Hu Z, Zhang Z, Doo E, et al. Hepatitis B virus $\times$ protein is both a substrate and a potential inhibitor of the proteasome complex. *J Virol.* 1999; 73:7231–7240. [PubMed: 10438810]
17. Wang Y, Reed M, Wang P, et al. p53 domains: identification and characterization of two autonomous DNA-binding regions. *Genes Dev.* 1993; 7:2575–2586. [PubMed: 8276240]
18. Lin Y, Nomura T, Yamashita T, et al. The transactivation and p53-interacting functions of hepatitis B virus $\times$ protein are mutually interfering but distinct. *Cancer Res.* 1997; 57:5137–5142. [PubMed: 9371515]
19. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet.* 2002; 31:339–346. [PubMed: 12149612]
20. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* 1999; 13:1501–1512. [PubMed: 10385618]
21. Xiong Y, Hannon GJ, Zhang H, et al. p21 is a universal inhibitor of cyclin kinases. *Nature.* 1993; 366:701–704. [PubMed: 8259214]
22. Bartek J, Lukas J. DNA damage checkpoints: from initiation to recovery or adaptation. *Curr Opin Cell Biol.* 2007; 19:238–245. [PubMed: 17303408]
23. Martin-Caballero J, Flores JM, Garcia-Palencia P, et al. Tumor susceptibility of p21(Waf1/Cip1)-deficient mice. *Cancer Res.* 2001; 61:6234–6238. [PubMed: 11507077]
24. Poole AJ, Heap D, Carroll RE, et al. Tumor suppressor functions for the Cdk inhibitor p21 in the mouse colon. *Oncogene.* 2004; 23:8128–8134. [PubMed: 15377995]
25. Yang W, Velcich A, Lozonschi I, et al. Inactivation of p21WAF1/cip1 enhances intestinal tumor formation in Muc2 $^{-/-}$ mice. *Am J Pathol.* 2005; 166:1239–1246. [PubMed: 15793302]
26. Carnero A, Beach DH. Absence of p21WAF1 cooperates with c-myc in bypassing Ras-induced senescence and enhances oncogenic cooperation. *Oncogene.* 2004; 23:6006–6011. [PubMed: 15195145]
27. Qiao L, Leach K, McKinsty R, et al. Hepatitis B virus $\times$ protein increases expression of p21(Cip-1/WAF1/MDA6) and p27(Kip-1) in primary mouse hepatocytes, leading to reduced cell cycle progression. *Hepatology.* 2001; 34:906–917. [PubMed: 11679961]
28. Park US, Park SK, Lee YI, et al. Hepatitis B virus-X protein upregulates the expression of p21waf1/cip1 and prolongs G1 $\rightarrow$ S transition via a p53-independent pathway in human hepatoma cells. *Oncogene.* 2000; 19:3384–3394. [PubMed: 10918595]
29. Kwun HJ, Jang KL. Natural variants of hepatitis B virus $\times$ protein have differential effects on the expression of cyclin-dependent kinase inhibitor p21 gene. *Nucleic Acids Res.* 2004; 32:2202–2213. [PubMed: 15107488]
30. Frescas D, Pagano M. Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. *Nat Rev Cancer.* 2008; 8:438–49. [PubMed: 18500245]
31. Hoeller D, Dikic I. Targeting the ubiquitin system in cancer therapy. *Nature.* 2009; 458:438–444. [PubMed: 19325623]
32. Bornstein G, Bloom J, Sitry-Shevah D, et al. Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. *J Biol Chem.* 2003; 278:25752–25757. [PubMed: 12730199]
33. Krude T. Initiation of human DNA replication in vitro using nuclei from cells arrested at an initiation-competent state. *J Biol Chem.* 2000; 275:13699–13707. [PubMed: 10788489]
34. Abbas T, Dutta A. p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer.* 2009; 9:400–414. [PubMed: 19440234]
35. Teufel A, Maass T, Strand S, et al. Liver-specific Ldb1 deletion results in enhanced liver cancer development. *J Hepatol.* 53:1078–1084. [PubMed: 20828852]
36. Charette N, De Saeger C, Lannoy V, et al. Salirasib inhibits the growth of hepatocarcinoma cell lines in vitro and tumor growth in vivo through ras and mTOR inhibition. *Mol Cancer.* 2010; 9:256–269. [PubMed: 20860815]
37. Senturk S, Mumcuoglu M, Gursay-Yuzugullu O, et al. Transforming growth factor-beta induces senescence in hepatocellular carcinoma cells and inhibits tumor growth. *Hepatology.* 52:966–974. [PubMed: 20583212]

38. Gao D, Inuzuka H, Tseng A, et al. Phosphorylation by Akt1 promotes cytoplasmic localization of Skp2 and impairs APC^{Cdh1}-mediated Skp2 destruction. *Nat Cell Biol.* 2009; 11:397–408. [PubMed: 19270695]
39. Calvisi DF, Ladu S, Pinna F, et al. SKP2 and CKS1 promote degradation of cell cycle regulators and are associated with hepatocellular carcinoma prognosis. *Gastroenterology.* 2009; 137:1816–1826. [PubMed: 19686743]

Figure 1. Promoter activity of HBx endogenous expressing plasmids and HBx protein expression. (A) HepG2 cells were transiently transfected with reporter constructs containing various lengths of the 5'-flanking region of the HBx gene upstream of a luciferase reporter gene. Bars indicate luciferase activity normalized to cells transfected with negative control pGL3-Basic vector. (B) HBx expression in cells transfected with WT or TA mutant HBx of genotypes A, B and C. Enh1: Enhancer 1; xp: × gene promoter.

Figure 2. p21 expression in WT and mutant HBx expressing cells. (A) Effects of WT and TA HBx of genotype A, B and C on p21 expression in HepG2 cells. (B) Effects of WT, TA, T1753A, T1768A or Combo mutant HBx of genotype C on p21 expression in HepG2 cells. (C) Effect of WT, TA and Combo mutant HBx on p21 expression in PHH.

Figure 3.

Effect of HBx on cell cycle progression. (A) Cell cycle distributions in HepG2 cells expressing WT and mutant HBx, with empty vector as a control. *Upper panels:* representative cell cycle plots. *Lower panel:* average percentage of cells in different phases of the cell cycle obtained from four analyses. Dot: S-phase in Combo mutant vs control Vector or TA, $P < .01$; asterisk: S-phase in Combo mutant vs WT HBx, $P < .001$. (B) Cotransfection of p21 abolished the S-phase stimulation by Combo mutant in Huh7 cells. Asterisk: p21 and Combo mutant cotransfection vs Combo mutant only, $P < .01$. (C) Dynamics of p21 expression and cell cycle progression in HBx transfected HepG2 cells. *Upper figure:* p21 expression at the indicated time points. *Lower figure:* cell cycle distributions at the same time points. (D) p21 silencing increased the proportion of S-phase in WT HBx expressing Huh7 cells. Asterisk: p21 siRNA vs nontargeting siRNA, $P < .01$. (E) Combo mutant accelerates S-phase progression. *Upper figure:* BrdU incorporation indices of synchronized HepG2 cells after serum release. Dot: Combo mutant vs TA or control Vector, $P < .05$; asterisk: Combo mutant vs WT, $P < .01$. *Lower figure:* BrdU incorporation indices of Combo mutant expressing Huh7 cells with and without p21 overexpression. Asterisk: p21 and Combo mutant cotransfection vs Combo mutant only, $P < .05$. Data

represent means $\pm$ SD from quadruplicate. (F) Effect of WT and mutant HBx on cyclin E expression in PHH.

Figure 4. Impact of HBx mutants on cell growth rate and colony formation. (A) Growth rates of HepG2 cells stably expressing HBx mutants were compared with that of cells expressing WT HBx and parental cells. Dot: Combo mutant vs TA or control Vector, $P < .05$; asterisk: Combo mutant vs WT, $P < .001$. (B) Soft agar anchorage-independent colony formation in NIH3T3 cells stably expressing a vector control, WT, TA mutant, and Combo mutant HBx. Dot: Combo mutant vs TA or control Vector, $P < .05$; asterisk: Combo mutant vs WT, $P < .01$.

Figure 5.

Core promoter mutations accelerate p21 protein turnover. (A) Effect of HBx on p21 protein stability/degradation. HepG2 cells were transfected transiently with WT, TA, or Combo mutant HBx, and then treated with 30μg/mL cycloheximide, followed by Western blot analysis. (B) p21 degradation occurs via the ubiquitin-proteasome pathway. At 48 hours posttransfection, HepG2 cells were treated with MG132 (25μmol/L) for 3 hrs before harvesting and then subjected to Western blot analysis. (C) p21 promoter activity is modulated by HBx expression. The indicated HBx expressing plasmids were cotransfected with p21 luciferase reporter construct. Dot: Combo mutant vs control Vector or TA, $P < .05$; asterisk: Combo mutant vs WT, $P < .001$. (D) Immunoblotting for p53 and p21 in WT and mutant HBx expressing HepG2 cells with p53 siRNA or scramble oligonucleotide (SC).

Figure 6.

Key role of SKP2 on WT and mutant HBx modulation of p21 expression and cell cycle progression. Detection of SKP2 protein in WT and mutant HBx expressing Huh7 cells (A) or primary human hepatocytes (B) by Western blot analysis. (C) Huh7 cells were transfected with control or SKP2-siRNA for 24 hrs and then transfected with WT, TA, and Combo mutant HBx, followed by Western blot (*Upper figure*) and determination of cell cycle distribution by FACS (*Lower figure*). Asterisk: S-phase proportion in SKP2 silencing cells vs nontargeting siRNA, $P < .01$. (D) BrdU incorporation indices of WT and mutant HBx expressing Huh7 cells with SKP2 knock-down. Asterisk: Combo mutant with SKP2 knockdown vs nontargeting siRNA, $P < .05$.