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# Hepatitis B virus X protein mutant upregulates CENP-A expression in hepatoma cells

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**Abstract.** The carcinogenic role of hepatitis B virus x protein (HBx) in hepatocellular carcinoma (HCC) remains largely unknown. Centromere protein A (CENP-A) has been found to be frequently overexpressed in HCC. In the present study, we aimed to investigate the role of HBx in regulating CENP-A activity in HCC carcinogenesis. CENP-A expression was examined and the HBx gene was sequenced in 20 HBsAg-positive HCC patients and corresponding non-cancerous liver tissues. The influence of HBx mutants on CENP-A expression in HepG2 cells was analyzed by a series of assays. We found that CENP-A expression was significantly elevated in HCC tissues. HBx deletion, especially the COOH-terminal deletion of HBx is a frequent event in HBV-associated HCC tissues. A positive correlation was found between CENP-A expression and HBx COOH mutation in HCC tissues. HBx mutant increased the expression of the CENP-A mRNA and protein compared with full-length HBx. However, HBx did not directly interact with CENP-A. It is concluded that overexpression of CENP-A is closely associated with HBx COOH mutation in HCC. HBx mutant can increase CENP-A expression, probably through a mechanism independent of their physical combination, and thereby it may represent a potential therapeutic strategy for HCC.

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the third leading cause of cancer-related mortality worldwide, especially in Asian countries (1-3). Chronic hepatitis B virus (HBV) infection is one of the main causes of HCC (2-5). Hepatitis B virus x protein (HBx), derived from hepadnavirus genomes, is a promiscuous transactivator, and it can cause aberrant expression of molecules involved in transcriptional regulation, signal transduction, cell cycle progress, protein degradation pathways, apoptosis, and genetic stability by directly or indirectly interacting with host factors (6-8). HBx deletion, especially the COOH-terminal deletion of HBx, was found in many HBV-infected HCC patients (9-12). Previous findings showed that different domains of HBx-COOH might represent various functions, and that the mutants may directly affect the proliferation and invasion of HCC by regulating the cell cycle. A major contributor of hepatocellular carcinogenesis is the deregulation of cell cycle progression, in particular the G1/S phase transition (13).

Centromere protein A (CENP-A) plays important roles in cell cycle regulation and genetic stability. Our previous work has shown that CENP-A was frequently overexpressed in HCC. RNAi-mediated CENP-A depletion suppressed HCC cell growth both *in vitro* and *in vivo*, blocked cell cycle progression at the G1 phase, and promoted apoptosis in HCC cells. The anticancer effects of CENP-A depletion are likely mediated by a large number of genes involved in cell cycle control and apoptosis, including CHK2, P21waf1, P27 Kip1, SKP2, MDM2, Bcl-2, and Bax (14,15). Furthermore, CENP-A overexpression was correlated with serum HBsAg states, tumor histological grade and P53 immunopositivity (16). In the present study, we aimed to investigate the relationship between CENP-A and HBx protein in HCC. We also studied the influence of HBx mutant on the CENP-A expression in HepG2 cells, in an attempt to probe into the underlying molecular mechanism for HBx action.

## Materials and methods

*Cell line and cell culture*. HepG2 (wild-type p53 and HBV negative) and Huh7 (characterized by p53 mutation with A:T→G:C at Codon 220) cell lines were cultured in Dulbecco's minimum essential medium (DMEM, Invitrogen Inc., Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin at 37˚C in a humidified incubator containing 5% $CO_2$.

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*Tissue samples*. Twenty HCC samples and the corresponding noncancerous liver tissues were obtained during surgeries in Changhai Hospital between January 2008 and December 2009 with prior patients' consent and approval from the Ethics Committee of the Second Military Medical University. The excised samples were obtained within one hour after the operation from tumor tissues and the corresponding adjacent noncancerous tissues 5-10 cm from the tumor. All the fresh tissues were cut into small pieces, snap-frozen in liquid nitrogen immediately, and stored at -80˚C until RNA or protein extraction. The patients were selected when met the following requirements: a) distinctive pathologic diagnosis of HCC according to the World Health Organization histological classification of tumors of the liver and intrahepatic bile ducts (2000) (17); b) receiving curative resection, defined as macroscopically complete removal of the neoplasm; and c) availability of detailed clinicopathologic data. Patients with preoperative anticancer treatment or with evidence of other malignancies were excluded. This study included 18 men and 2 women, with an age range of 31-69 years (median: 50 years), and all patients were positive for serum hepatitis B surface antigen (HBsAg). The median tumor size was 5.5 cm (2.0-12.5 cm), with 3 well differentiated ones, 14 moderately differentiated ones, and 3 poorly differentiated ones. Of the 20 patients, six had evidence of intrahepatic metastasis (portal vein invasion and/or intrahepatic dissemination). Sixteen HCC patients also had liver cirrhosis and the other 4 had chronic hepatitis. All patients were negative for serum HCV and HIV.

*Polymerase chain reaction and sequence analysis of HBx*. Genomic DNA was extracted from the 20 frozen HCC tissues and corresponding noncancerous liver tissue using the standard phenol/chloroform extraction and ethanol precipitation method. To amplify the integrated HBx sequences from tissues, we used an HBx-Alu PCR-based approach (18). The sequences of the primers used are: 5'-TGC CAA GTG TTT GCT GAC GC-3' (HBV 1176-1195, AY220699), 5'-AAG GAA AGA AGT CAG AAG G-3' (HBV 1960-1978) (6). The fragment size is 803 bp. After denaturation at 94˚C for 2 min, DNA amplification was performed for 36 cycles at 94˚C for 30 sec, at 53˚C for 60 sec, and at 72˚C for 60 sec; with a final extension at 72˚C for 10 min and stored 4˚C. PCR results were identified by electrophoresis on a 1% agarose gel stained with ethidium bromide.

*Immunohistochemistry*. Immunohistochemistry was performed by SP-9000 Histostain™-plus kit (Zhongshan Golden Bridge Biotech, Beijing, China) as previously described (19). The primary antibodies included anti-CENP-A (ab13939, 1:100, Abcam, Cambridge, UK), and anti-Hepatitis B virus X antigen (HBxAg, ab235, Abcam; 1:500). We defined that a <10% represented low, and >10% represented high levels of CENP-A expression (16).

*Plasmid construction, transfection and detection*. Plasmid construction of full-length HBx or HBx mutant with 40 amino acids deleted at the COOH-terminus (namely, HBx3'-40) and detection of stable transfection were performed as previously described.

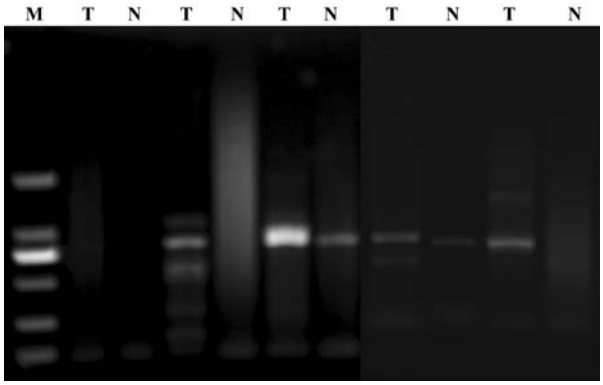


Figure 1. PCR amplification products of HBx-DNA. The fragment sizes of PCR products range from 150 to 800 bp. M, Marker; T, Tumor; N, Non-tumor.

*RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR), and qPCR*. RT-PCR and qPCR analysis were performed as previously described (16,20). Total RNA was extracted from cells or tissues with an RNeasy mini kit (Qiagen, Germany) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from total RNA with the First-Strand cDNA Synthesis Kit for RT-PCR (Roche, Mannheim, Germany). RT-PCR amplification was done using Taq DNA Polymerase (AmpliTaq Gold; Roche Molecular Systems, Pleasanton, CA) at the following conditions: one cycle at 94˚C for 5 min, 30 cycles at 95˚C for 30 sec, 58˚C for 45 sec, and 72˚C for 1.5 min, and one cycle at 72˚C for 10 min. CENP-A cDNA was amplified with primers: forward 5'-ACAAGGTTGGCTAAAGGA-3' and reverse 5'-ATGCTTCTGCTGCCTCTT-3' (178 bp). For controls, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified in a parallel reaction, with the following primers: 5'-TCCACCACCCTGTTGCTGTA-3' and 5'-ACCACAGTCCATGCCATCAC-3' (452 bp). RT-PCR products were separated by electrophoresis on 1.5% agarose gels. The qPCR was performed using SYBR-green detection of PCR products in real time with the Light Cycler (Roche Diagnostics, Meylan, France). The cycling conditions were as follows: initial denaturation at 95˚C for 10 min, and then 45 cycles of denaturation at 95˚C for 5 sec, annealing at 62˚C for 20 sec, and elongation at 72˚C for 15 sec. As an internal quantitative control, β-actin gene expression was determined with the primers: 5'-GAGCGGGAAATCGTGCGTGACATT-3' and 5'-GATGGAGTTGAAGGTAGTTTCGTG-3' (234 bp). Gene expression was analyzed using the Light Cycler software version 3.5 (Roche Diagnostics), and the ratios of CENP-A to β-actin represented normalized relative levels of CENP-A expression (21). A no-template negative control was also included in each experiment. Analyses of all samples were carried out in triplicate, and the mean values were calculated.

*Protein extraction and Western blot analysis*. Primary antibodies employed were: anti-CENP-A (ab13939, Abcam, 1:500), anti-Hepatitis B virus X antigen (ab235, Abcam; 1:500), and anti-GAPDH (Santa Cruz Biotech, Santa Cruz, CA; 1:2000). Protein isolation from the culture cells or tissues and Western blot analysis were carried out as previously described (19). Briefly, culture cells or tissues were lysed

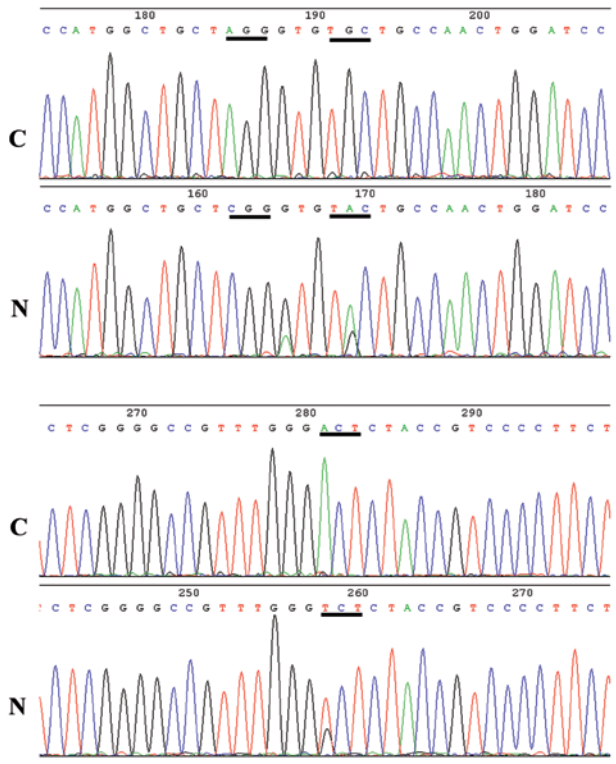


Figure 2. (Upper panel) AGG→CGG sense mutations at 4aa and (lower panel) TGC→TAC and ACT→TCT mis-sense mutations at 6aa and 36aa in non-cancerous liver tissues. C, Control; N, Non-tumor.

in buffer containing 10 mmol/l Tris (pH 7.4), 1% sodium dodecyl sulfate (SDS), 1 mmol/l sodium orthovanadate, and complete protease inhibitors (Roche). The extraction was accomplished using the Klose method as described previously (22,23). Samples of the lysates (50 $\mu$g) were then separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA), and blotted with the primary antibodies. After incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotech), the blots were visualized with enhanced chemiluminescence (sc-2048, Santa Cruz Biotech). The intensity of each band was measured using a Fluor-S MultiImager and Quantity-One software (Bio-Rad, Hercules, CA).

*Immunoprecipitation*. All immunoprecipitation procedures were carried out at 4˚C. Cells were harvested and washed twice with PBS before lysis. Cells were lysed in lysis buffer, and the lysates were then incubated with the appropriate antibody for 1 h, and were subsequently incubated with protein A-Sepharose beads for 1 h. The protein-antibody complexes on beads were subjected to Western blot analysis after separation by SDS-PAGE.

*Statistical analysis*. Data were presented as the means ± SD. All statistical calculations were carried out using SPSS.11 software (SPSS, Chicago, IL). The relationship between CENP-A expression, HBsAg and HBx COOH-terminal deletion of HCCs was statistically analyzed by using the Pearson Chi-square test. Spearman's bivariate correlation was used to determine whether there is a positive or negative correlation between the HBx mutation and CENP-A expression levels. The difference among the means of multiple groups was analyzed by one-way analysis of variance (ANOVA) followed by the Tukey test. A correlation coefficient >0.5 or <-0.5 was considered significant for Spearman's correlation. A difference was defined as significant at $P<0.05$.

## Results

*HBx gene sequencing in hepatocellular carcinoma tissues from HBsAg-positive patients*. To confirm the HBx deletion mutation, HBx gene was amplified and sequenced from the genomic DNA of 20 HBsAg-positive HCC patients. The fragment sizes of PCR products were 150-800 bp (Fig. 1). The results of sequencing were compared with the known sequences in the GenBank database using the BLAST programs. As we previously described (24), the frequencies of HBx point mutations were significantly lower in HCC tissues than in the corresponding noncancerous liver tissues (7/20 vs. 15/20, $P<0.001$). In 20 available non-tumorous livers, sequencing results revealed 42 different point mutation patterns, including 24 missense mutations, 16 sense mutations and 2 nonsense mutations. Besides the loci with the highest frequency of mutation at 67aa (15/20, all were gga→ggt sense mutations) and 127aa (12/20, att→act missense mutations), one agg→cgg sense mutation at 4aa and 12 missense mutations (eight tgc→tac at 6aa, and four act→tct 36aa) were found (Fig. 2).

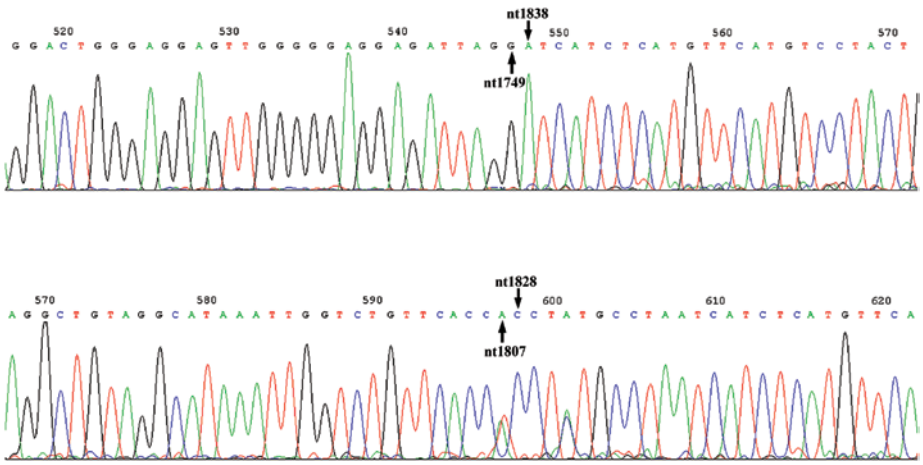


Figure 3. Sequencing of HBx deletion mutation in HCC.

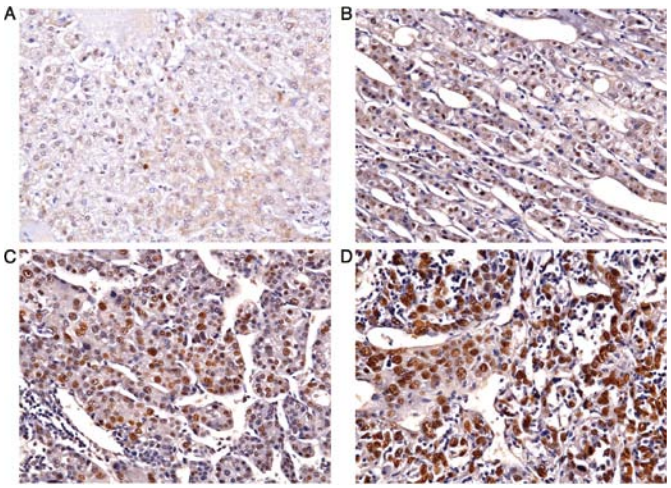


Figure 4. Immunohistochemistry for CENP-A in HCC and adjacent non-cancerous liver tissues. Representative CENP-A staining in (A) nontumorous liver tissue, and in (B) well-differentiated, (C) moderately-differentiated, and (D) poorly-differentiated HCC tissues. The CENP-A staining is predominantly nuclear in the samples examined, with concurrent diffused cytoplasmic staining in poorly-differentiated HCC.

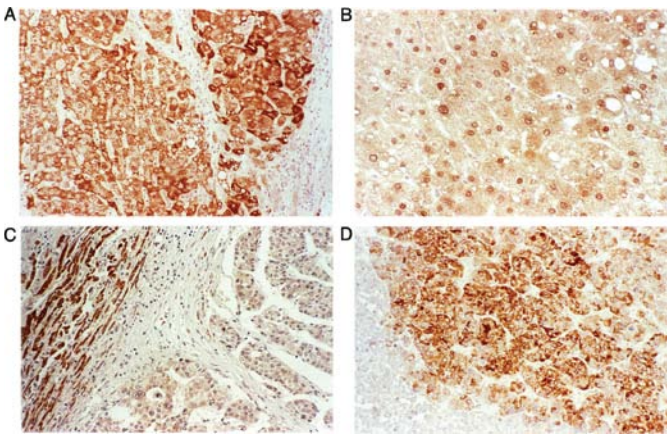


Figure 5. Immunohistochemistry for HBxAg in HCC and adjacent non-cancerous liver tissues. Representative HBxAg staining of (A) diffused cytoplasmic staining and (B) predominantly nuclear staining in adjacent non-cancerous liver tissues, and in (C) negative and (D) diffused cytoplasmic staining in HCC.

In contrast, the frequencies of HBx deletion mutation in HCC tissuses were significantly higher than in the corresponding noncancerous liver tissues (8/20 vs. 1/20, P<0.001). Among of them, the COOH-terminal deletion of HBx was found in 7 HCC samples, with the size ranging 4aa-150aa (Fig. 3). Therefore, COOH-terminal deletion is a major feature of HBx identified in tumor tissue. In addition, a specific integration of HBx at GA-rich region of 17p13 locus (repeat region 56047...56210/ rpt_family = 'GA-rich') was also found in 6 of the 14 HCC patients in our previous study (24).

*Association between CENP-A expression and HBx COOH-terminal deletion in HCC patients*. Immunohistochemistry examination with anti-CENP-A and HBxAg antibodies was performed on 20 pairs of paraffin-embedded human HCC specimens and adjacent nontumorous liver tissues. A low frequency of the surrounding nontumorous tissues (2/20, 10%) displayed rare positive nuclei (Fig. 4A), while a markedly higher proportion of HCC samples (11/20, 55%) showed positive CENP-A immunostaining. CENP-A expression appeared to increase with the differentiation grade of HCC cells from well differentiated to poorly differentiated (Fig. 4B-D).

Strongly positive HBxAg immunoreactivity was observed in 16 of 20 (80%) non-cancerous liver tissues. In hepatocytes, the HBxAg protein was strongly and uniformly stained in both the cytoplasm and/or nucleus (Fig. 5A); all of the surrounding connective tissues, including blood vessels and bile ducts, were negative for HBxAg protein. The HCC cells

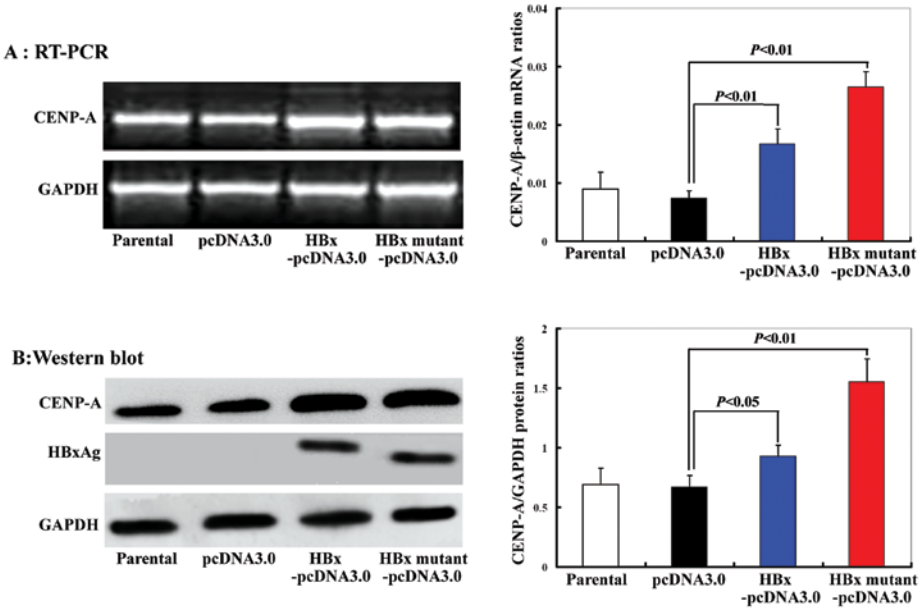


Figure 6. Analysis of CENP-A and HBxAg expression in Huh 7 cells transfected with HBx mutant- or full-length HBx-expressing plasmids, as described in Materials and methods. (A) RT-PCR (left) and qPCR (right) measurement of CENP-A transcript in cells transfected with HBx mutant, full-length HBx or pcDNA3.0 empty vector. (B) Left: representative Western blots for CENP-A and HBxAg in cells treated as in (A). Right: quantification of the blotting shown in the left panel. Student's t-test; n=3.

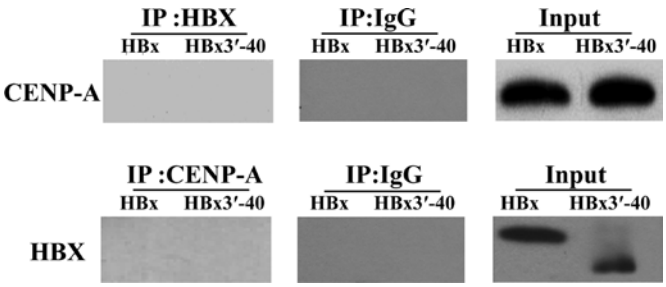


Figure 7. HBx physically interacts with CENP-A in HepG2 cells. HBx or HBx mutant was transfected into HepG2 cells as indicated and cell lysates were then immunoprecipitated using anti-HBxAg or -CENP-A antibody. The immunoprecipitates were examined by Western blot analysis using anti-HBxAg or -CENP-A antibody. Input represented 10% of cell lysates used in the Co-IP experiment. HBx did not directly interact with CENP-A.

Table I. Associations between expression of CENP-A, HBxAg and HBx COOH-terminal deletion in HCC patients (n=20).

| Groups | CENP-A | | P |
|---|---|---|---|
| | + | - | |
| HBxAg | | | |
| + | 7 | 5 | >0.05 |
| - | 4 | 4 | |
| HBx COOH-terminal deletion | | | |
| + | 8 | 0 | <0.001 |
| - | 3 | 9 | |

were stained in various patterns, being diffusely to focally stained in 12 patients (60%), and negatively stained in 8 patients (40%) (Fig. 5B).

We also investigated the status of HBx mutations and their correlation with CENP-A expression in HCC tissues. Spearman's Chi-square analysis showed that there was a positive correlation between CENP-A expression and the HBx COOH mutation in HCC tissues (P<0.001). However, no obviously correlation was found between the upexpression of CENP-A protein and HBxAg immunoreactivity in HCC tissues (Table I).

Our previous results showed that HBx3'-40 amino acid deletion mutants, compared with the full-length HBx, resulted in an increase in cellular proliferation, focus formation, tumorigenicity, and invasive growth and metastasis through promoting cell cycle from G0/G1 to the S phase. The expression of P53, p21WAF1, p14ARF, and MDM2 proteins was affected by HBx mutant. Moreover, we investigated the effects of HBx and its COOH mutant on CENP-A expression. As shown in Fig. 6, HBx mutant increased expression of the CENP-A mRNA and protein compared with full-length HBx.

*HBx physically interacts with CENP-A*. To test whether HBx interacts with CENP-A, HepG2 cells were transfected with HBx or HBx mutant, and then subjected to co-immunoprecipitation (Co-IP) experiment. The total cell lysate of HepG2 cells was immunoprecipitated with HBx antibody, and CENP-A antibody was used in Western blot analysis to examine the existence of CENP-A in the HBx immunoprecipitates. As shown in Fig. 7, HBx did not directly interact with CENP-A.

## Discussion

CENP-A plays a central role in directing kinetochore assembly, which is indispensable for equal chromosome segregation (25-27). In this study, we further confirm and extend our previous findings (14,15) that CENP-A expression is significantly elevated in HCC tissues. RNAi-mediated CENP-A depletion suppresses HCC cell growth both *in vitro* and *in vivo*, blocks cell-cycle progression at the G1 phase, and promotes apoptosis. The anticancer effects of CENP-A depletion are likely mediated by a large number of genes involved in cell-cycle control and apoptosis, including CHK2, P21waf1, P27 Kip1, SKP2, MDM2, Bcl-2, and Bax (16). These results suggest that aberrant expression of CENP-A may be an important etiologic factor of HCC, and it may provide a potential therapeutic target for HCC.

HBx is known to play an important role in hepatocarcinogenesis, although the exact functions and molecular mechanisms are not well understood. SiRNA effectively suppressed HBx gene expression in HCC cells and inhibited HCC cell proliferation. Reduced HBx expression results in enhanced susceptibility of cells to anticancer drugs and induced proliferation suppression and apoptosis (28). HBx can increase SMYD3 expression, and SMYD3 upregulates c-myc in hepatoma; therefore we suggest that SMYD3-c-myc might be one of most important pathways in the development and progression of HBx-related HCC (29). HBx also plays a role in regulating a series of cell-signaling cascades, most notably in the Ras- and Raf-induced mitogen-activated protein kinase pathways, and in inactivation of tumor suppressive genes through promoter hypermethylation (30). Previous studies have demonstrated that HBx deletion, especially the COOH-terminal deletion of HBx, is a frequent event in HBV-associated HCC tissues (11,12,24).

Deletions in the HBx COOH-terminal abolished the important roles wild-type HBx COOH-terminal played in controlling cell proliferation, differentiation, and transcriptional activity (6). Ours and other previous findings have shown that different domains at HBx-COOH may represent various mechanisms, and the mutations may directly affect the proliferation and invasion by regulating the cell cycle and inducing the expression of $p21^{WAF1}$, $p14^{ARF}$ and MDM2 (13). This supports the hypothesis that deleted forms of HBx may contribute to liver carcinogenesis (13,30-32). Our previous findings also demonstrated that CENP-A overexpression was correlated with serum HBsAg states in HCC patients (16). In this study, we identified a positive correlation between the expression of CENP-A immunoreactivity and the HBx COOH mutation in HCC tissues. The COOH-terminal deletion is a major feature of HBx in HCC tissues. Expression of the CENP-A mRNA and protein levels was increased in HBx mutant compared with full-length HBx, suggesting HBx may affect HCC cell proliferation via multiple regulators of cell-cycle progression.

Similar to several other viral oncoproteins, HBx protein is a promiscuous transactivator, with the transcriptional activity mediated via protein-protein interaction. To dissect the effects

of HBx COOH mutation on CENP expression in HCC, we employed co-immunoprecipitation approach to assess the interaction of HBx with CENP-A in HepG2 cells. However, we found that HBx did not directly interact with CENP-A, suggesting that HBx may affect CENP-A via an unknown mechanism; further investigations are needed to test this hypothesis.

In summary, we further confirm that CENP-A expression is significantly elevated in HCC. The COOH-terminal deletion is a major feature of HBx in HCC tissues. Overexpression of CENP-A is closely associated with HBx COOH mutation in HCC. HBx mutant increases CENP-A expression likely through a mechanism independent of their physical combination and thereby represents a potential therapeutic strategy for this malignancy.

## Acknowledgements

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