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Full-length article

Identification of a natural mutant of HBV X protein truncated 27 amino acids at the COOH terminal and its effect on liver cell proliferation[1]

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Key words

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Abstract

Aim: To identify mutants of the hepatitis B virus (HBV) X (HBx) gene and investigate the effect of the natural mutant on liver cell proliferation. **Methods:** We identified natural mutants of the HBx gene from 188 sera and 48 tissues of Chinese patients infected with HBV by PCR, respectively. Based on the identification of the mutants of HBx gene, we cloned the fragments of the mutants into the pcDNA3 vector. The biological activities of the mutants were investigated. **Results:** We identified a natural mutant of the HBx gene with deletion from 382 to 401 base pairs from 3 sera out of 188 patients, which resulted in the expression deletion of the HBx protein from the 128th amino acid at the COOH terminal. The similar mutant with deletion from 382 base pair at the COOH terminal was identified from 5 cases of genomes out of 48 hepatocellular carcinoma tissues. Regarding the biological activities of the mutant, we found that the mutant of the HBx protein failed to induce apoptosis by transient transfection, but promoted proliferation of human liver immortalized L-O2 cells by stable transfection, compared with the wild-type HBx protein. The data showed that the proliferation of the mutant stably-transfected L-O2-X-Sera cells and fragment stably-transfected L-O2-XΔ127 cells was enhanced by the BrdU incorporation assay and flow cytometry analysis. Luciferase reporter gene assay showed that the transcriptional activities of NF-κB, survivin, and human telomerase reverse transcriptase were upregulated, and Western blot analysis revealed that the expression levels of c-Myc and proliferating cell nuclear antigen (PCNA) were upregulated in the cells. **Conclusion:** Our findings suggest that the natural HBx mutant truncated 27 amino acids at the COOH terminal promotes cell proliferation.

Introduction

Chronic hepatitis B virus (HBV) infection plays a major causative role in the development of hepatocellular carcinoma (HCC)[1,2]. In China, HCC is the second most common fatal cancer. HBV, which belongs to the hepadnaviridae family, causes both acute and chronic infection of the liver. The HBV genome consists of a circular, partially double-stranded DNA molecule of 3.2 kb, which contains 4 genes named S, C, P, and X genes. The HBV X (HBx) gene is the smallest one with a length of 465 base pairs encoding a 154 amino acid protein with a molecular weight of 17 kDa, which plays a crucial role in the development of HCC[2]. The HBx gene was initially identified as a viral transcriptional transactivator, which can interact with a wide variety of viral and cellular regulatory elements, such as nuclear transcription factors and basal transcriptional machinery of host RNA polymerases[1,3]. During the course of HBV infection, the integration of the HBV genome into the host genome occurs frequently[3]; mutations of HBV DNA are found in the integration. These mutations may reflect the function of the virus to adjust itself to the host immunity, which may cause the virus to inhibit the host for a long time after infection,

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and may precede the development of HCC. Several studies of HBx mutations in patients with liver diseases have been reported previously. Point mutation in the HBx gene, especially the double substitution (nt1762 A-T, nt1764 G-A), leading to Lys-Met130 and Val-Ile131, occurs more frequently in patients with liver cirrhosis and/or HCC than in patients with chronic hepatitis B[4]. An insert mutation at nt204 (insert AGGCCC) accompanied with nt260 (G→A) and nt264 (G/C/T→A) was detected most frequently in tissues and sera samples from HCC patients[5]. There are also some reports of deletion mutation of the HBx gene from the tumor tissues of HCC, which may lead to the expression of the COOH terminal-truncated HBx protein[6]. However, the reported biological impacts of the mutant HBx protein are conflicting. This may be related to the different cell lines used in the experiments and/or the different mutant patterns of the HBx gene.

In the present study, we identified a natural similar mutant of the HBx gene from the sera of Chinese patients with HBV infection by sequencing, as well as tissues of Chinese patients with liver cancer, followed by an investigation of the biological functions of the mutant. We found that the mutant of the HBx protein failed to induce apoptosis, but promoted more cell growth compared with the wild-type HBx protein.

Materials and methods

Patient samples The samples of sera were taken from 188 cases of patients with liver diseases, including 42 chronic hepatitis B patients, 94 liver cirrhosis patients, and 52 HCC patients (137 males and 51 females aged 18~81 years, with an average age of 47). The samples were obtained from Tianjin Third Central Hospital, Tianjin, China. All patients had a record of HBV markers according to hospital data. We previously examined the HBV x antigen (HBxAg) and antibody to HBxAg in the sera of this group patients[7]. Samples of liver tumor tissues were taken from 48 cases of patients with HCC from Tianjin First Central Hospital, Tianjin, China (43 males and 5 females aged 21–70 years, with an average age of 51.9). All had undergone total or subtotal hepatectomies. All of the tumor tissue samples were frozen immediately after surgical resection and stored in liquid nitrogen. All of the patients had a history of HBV infection according to the hospital data.

DNA extraction from sera and PCR An equal volume (20 μL) of serum and DNA extract solution (Zhongshan Da'an Gene, Guangzhou, China) were mixed together and boiled for 10 min, followed by stilled at 4 °C for 12 h, and then centrifuged at 10 000 r/min for 5 min; 5 μL supernatant was used for the PCR reaction. We used a nested PCR program[8] to amplify the HBx gene from the sera of patients. The outer primers used were 5' GTTTGCTGACGCA ACCCCC3' (nt1182–nt1200) and 5' CAATGTCCATGCCCC AAAGC3' (nt1891– nt1910), and the inner primers were 5' GATCCATACTGCGGAACT CC3' (nt1263–nt1282) and 5' AGCTTGGAGGCTTGAACAGT3' (nt1859–nt1878). The PCR products were corroborated by 1.5% agarose gel electrophoresis with ethidium bromide. Then the purified PCR products were cloned into the vector pMD18-T (TAKARA, Dalian, China) and sequenced by Sunbio Bio-Technical (Beijing, China).

DNA extraction from HCC tissues and Alu–PCR To identify the integrated HBx gene in HCC tissues, an HBx-Alu-PCR approach were used[9]. DNA was extracted from HCC tissues by proteinase K digestion followed by phenol/chloroform extraction, as previously described[10]. A PCR-based technique (Alu–PCR) was employed by using specific primers for a human Alu sequence and the HBx sequence to effectively detect the HBX–host junction, as described previously[11,12]. The first 10 cycles of amplification were undertaken in a thermal cycler in a final volume of 50 μL containing 100 ng genomic DNA as a template, 10 pmol/L Alu primer, 100 pmol/L HBx primer, and 2.6 U *Taq* DNA-Two DNA polymerase mixed with an Expand™ High Fidelity assay kit (Roche, Mannheim, Germany). The reaction was carried out as a ''hot start'' PCR using the *Taq* start antibody (Clontech, Palo Alto, CA, USA). The cycling conditions consisted of denaturation for 30 s at 94 °C, annealing for 30 s at 59 °C, and extension for 3 min at 70 °C, with an initial denaturation period of 1 min at 94 °C. One unit of uracil DNA glycosylase (GIBCO/BRL, Paisley, UK) was then added to each of the tubes, and the tubes were incubated for 30 min at 37 °C. After heating for 10 min at 94 °C to break the DNA strands at the apurinic dUTP sites, 10 pmol/L of each primer was added for the next amplification. The "touchdown" PCR technique was employed for this amplification[13]. Denaturation was carried out at 94 °C for 30 s and extension at 70 °C for 3 min. The annealing step was started at 65 °C for 30 s; the temperature was then reduced by 1 °C every second cycle until a temperature of 55 °C was reached, at which point 20 cycles had been carried out. The final extension was carried out for 8 min at 72 °C. Thus, a total of 40 cycles were made, and 1 mL of the product was subjected to hemi-nested PCR with the initial primers to obtain discrete bands. Amplified PCR products were analyzed by electrophoresis on 1% agarose gel. Then the purified PCR products were cloned into the pMD18-T vector (TAKARA, Dalian, China), and the posi-

tive clones were sequenced by Sunbio Bio-Technical (Beijing, China).

Plasmid construction To construct the eukaryotic expression vector of the mutant HBx gene, the pMD18-T plasmids cloning the mutant HBx gene from 3 sera of patients were digested by *Eco*RI and *Xho*I, and the mutant HBx gene fragment was ligated into the pcDNA3 vector, respectively. The mutant HBx gene from patient 8-27-2 was successfully cloned into the pcDNA3 vector, termed pcDNA3-X-Sera, followed by confirmation of sequencing. The fragment of the HBx gene that expresses the protein truncated 27 amino acids at the COOH terminal was generated by PCR using the pCMV-X plasmid as a template. The primers, including 5'-CAGAATTCATGGCTGCTAGGCTGTGC-3' and 5'-TACTCGAGAATCTCCTCCCCCAACT-3', were used. The PCR products were subcloned into the pcDNA3 vector, termed pcDNA3-XΔ127, followed by the confirmation of sequencing.

Cells culture and transfection The human liver cell line L-O2 (Nanjing KeyGen Biotech, Nanjing, China), originating from normal human liver tissues that had been immortalized by the stable transfection of human telomerase reverse transcriptase (hTERT) gene, had been previously used[14–17]. The L-O2 cells were cultured in RPMI-1640 medium (GIBCO, Carlsbad, California, USA) supplemented with 10% heat-inactivated fetal calf serum and penicillin (100 U/mL) and streptomycin (100 mg/mL) in a 5% CO_2 atmosphere at 37 °C. The detailed procedures of transfection were followed accordingly[14]. The L-O2 cells were transiently transfected with 2 μg plasmids, such as the pcDNA3 empty vector, pcDNA3-X-Sera (from patient 8-27-2), pcDNA3-XΔ127, and pCMV-X, respectively, by using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instruction. Transfection efficiency in the cells was monitored by cotransfection of 0.2 μg pEGFP-C2 plasmid, which expresses the green fluorescence protein. After 48 h transfection, flow cytometry analysis was performed to detect apoptosis and cell cycle. Meanwhile, the expression of the HBx protein was examined by Western blot analysis in the transfected L-O2 cells. To construct cell lines stably expressing the wild-type or mutant HBx gene, G418 was added to the medium to a final concentration of 600 μg/mL after 48 h transfection. The cell lines that were stably transfected the pCMV-X (termed L-O2-X), pcDNA3-XΔ127 (termed L-O2-XΔ127), pcDNA3-X-Sera (termed L-O2-X-Sera), and pcDNA3 empty vector (termed L-O2-pcDNA3) plasmids, respectively, were examined for the presence of wild-type or mutant HBx gene in the host genome by PCR.

Flow cytometry analysis The detailed procedures were followed accordingly[14]. The stable transfected cell lines, including L-O2-X, L-O2-XΔ127, L-O2-X-Sera, and L-O2-pcDNA3, were examined by a FACScan flow cytometer (Becton, Dickinson, San Jose, CA, USA), followed by the examination of cell proliferation. The cell proliferative index (PI) is the sum of the S and G_2/M phase activities of the cell cycle expressed as a fraction of the total cell population, that is, $PI=([S+G_2/M]/[G_0/G_1+S+G_2/M])\times 100$[14]. The experiment was repeated 3 times.

BrdU labeling and immunofluorescent staining The detailed procedures were followed accordingly[14]. The 5'-bromodeoxyuridine (BrdU) labeling index was assessed by point counting through a Nikon TE200 inverted microscope (Nikon, Tokyo, Japan) using a 40× objective lens. Propidium iodine (Sigma, St Louis, MO USA) staining for nuclei in 50 μg/mL was used as a control to all cells in each group.

Luciferase reporter gene assays The luciferase reporters used included pGL3-NF-κB, pGL3-hTERT, pGL3-survivin, pGL3-basic, and renilla luciferase reporter vector pRL-TK. For transient transfections, the L-O2 cells were collected and plated in 24-well plates at 0.3×10^5 cells per well. The L-O2 cells were transiently transfected with 0.3 μg plasmids, including the pcDNA3 empty vector, pcDNA3-XΔ127, pcDNA3-X-Sera, and pCMV-X, respectively, by using Lipofectamine 2000 according to the manufacturer's instruction; each group of cells was cotransfected with 0.3 μg pGL3-NF-κB, or pGL3-hTERT, or pGL3-survivin, or pGL3-basic plasmids, respectively. Each well of cells was cotransfected with 10 ng pRL-TK. The cells were harvested after 24 h. The cells were lysed in 1× passive lysis buffer, and luciferase activity was determined by using the Dual luciferase reporter assay system (Promega, San Luis Obispo, CA USA) on a luminometer (TD-20/20, Sunnyvale CA, USA) according to the manufacturer's instructions. The pcDNA3 empty vector, pGL3-basic plasmid, and mock were used as controls.

Western blot analysis For the identification of the tumor-related proteins, the detailed procedures of the Western blot analysis were followed in the transfected L-O2 cells accordingly[14]. The primary antibodies were mouse anti-c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:500 dilution), rabbit anti-proliferating cell nuclear antigen (PCNA; NeoMarkers, Fremont, CA, USA, 1:1000 dilution), rabbit anti-HBx (1:150 dilution)[24], and mouse anti-β-actin (Sigma, USA, 1:20 000 dilution).

Statistical analysis The statistical analysis was performed using SigmaPlot 2001 (Systat Software, Richmond, CA, USA, http://www.systat.com). Statistical significance was assessed by comparing mean vales (±SD) using

Student's *t*-test or χ^2-test.

Results

Identification of a natural HBx gene mutant The HBx genes in the sera of patients with HBV-related liver diseases were amplified by PCR. The PCR products were analyzed by agarose gel electrophoresis (Figure 1A). The sequences showed that a natural mutant of HBx gene was identified in the sera from 1 case of chronic hepatitis B and 2 cases of liver cirrhosis. The 3 mutants from the sera were similar to each other in sequence. In the case of patient 8-27-2, a fragment deletion at nt382–400 of the x gene (codons 128–133 amino acids) was found. In other 2 cases (patients 8-30-15 and 9-6-8), a fragment deletion at nt382-401 of the x gene was observed. Figure 1B shows the matched sequences that demonstrate a new stop codon TAG formation observed in the downstream of each mutant after the fragment deletion. The clinical examination of HBV in the sera from the 3 patients is shown in Table[7]. The clinical examination of HBV in 5 out of 48 cases of HCC tissues is shown in Table 2.

To amplify the HBx gene and its insertion from the tissues of HBV-related HCC patients, a HBV–Alu–PCR technique was performed. The HBx gene was successfully amplified from 5 out of 48 cases of tumor tissues. Alu–PCR products were analyzed by agarose gel electrophoresis (Figure 1C). The sequences showed that the fragment of the HBx gene inserted in the genome after nt382 was truncated at the 3' end.

Truncated HBx protein at COOH terminal loses capability of inducing apoptosis After the transient transfection, the flow cytometry analysis showed that the wild-type HBx protein was able to induce apoptosis (19.65%; Figure 2A, but the mutants of the HBx protein, such as pcDNA3-XΔ127 and pcDNA3-X-Sera, failed to induce apoptosis (Figure 2A), suggesting that the HBx mutants lost the ability to induce apoptosis. The expression of the HBx protein was shown in the transfected L-O2 cells (Figure 2A).

Truncated HBx protein at COOH terminal enhances L-O2 cell growth The stably mutant-transfected L-O2 cells or stably fragment-transfected L-O2 cells were

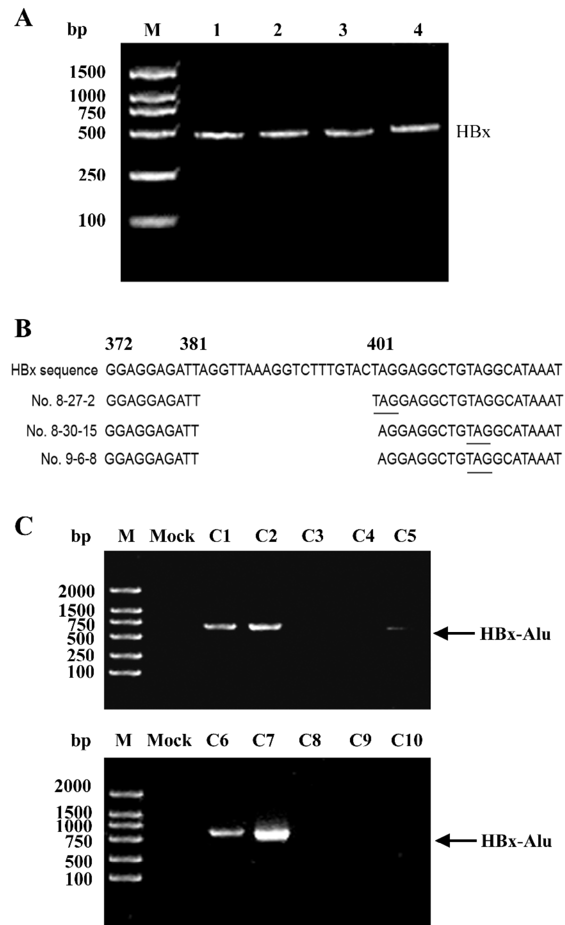

Figure 1. Identification of HBx gene mutants from sera and HCC tissues of Chinese patients with HBV infection. (A) PCR products were corroborated by 1.5% agarose gel electrophoresis stained with ethidium bromide. Lanes 1–3, PCR products of HBx gene from the sera of patients; lane 4, wild-type HBx gene in PCMV-X plasmid as a positive control. (B) sequences of HBx mutants from sera of 3 cases of patients were matched to HBx sequence, respectively. Underlined nucleotides show the new stop codon generated in the HBx mutants. (C) identification of HBx gene integrated in the cell genome from hepatoma tissues by Alu–PCR. M, DL2000 DNA marker; mock, negative control; C1, C2, C5, C6, and C7 were PCR products from the HBx gene integrated in the genome of HCC tissues.

Table 1. Clinical examination of hepatitis B virus in sera from patients.

No.	Sex	Age	Diagnosis	HBsAg	Anti-HBs	HBeAg	Anti-HBe	Anti-HBc	HBxAg	Anti-HBx
8-27-2	M	37	CHB	+	-	+	+	+	-	+
8-30-15	M	55	LC	+	-	+	-	+	+	+
9-6-8	F	60	LC	+	-	+	-	+	-	+

Table 2. Clinical examination of hepatitis B virus in 5 out of 48 cases of HCC tissues.

No.	Sex	Age	HBsAg	Anti-HBs	HBeAg	Anti-HBe	Anti-HBc
1	M	43	+	-	-	+	+
2	M	42	+	-	-	+	+
5	M	42	+	-	-	+	+
6	M	57	+	-	-	+	+
7	M	59	+	-	-	+	+

established, and termed L-O2-X-Sera or L-O2-XΔ127, respectively, followed by the investigation of the effect of the HBx mutants on the proliferation of the L-O2 cells by flow cytometry analysis and BrdU incorporation analysis. The results showed that the percentage of cells both in S phase and in G_2/M phase significantly increased in both L-O2-XΔ127 cells and L-O2-X-Sera cells than the controls, suggesting that the overexpression of the truncated HBx protein at the COOH terminal led to significantly increased cell proliferation according to the PI (P<0.01 *vs* control or L-O2-X, χ^2-test; Figure 2B). The BrdU incorporation analysis showed that the induction of DNA synthesis by the truncated HBx gene was increased in L-O2-XΔ127 and L-O2-X-Sera cells (P<0.05 *vs* control or L-O2-X; Student's *t*-test; Figure 2C, 2D), suggesting that the HBx mutant enhanced L-O2 cell proliferation.

Truncated HBx protein at COOH terminal upregulates transcriptional activity of NF-κB, survivin, and hTERT The L-O2 cells were transiently transfected with the wild-type HBx gene, mutant HBx gene, or the empty vector, respectively. The transcription levels of NF-κB, survivin, and hTERT were detected by the luciferase reporter gene assay. The results indicated that both the mutant and wild-type HBx proteins were significantly able to stimulate the promoter transcriptional activities of NF-κB, survivin, and hTERT in L-O2 cells compared to the controls (P<0.01 *vs* control, such as mock, pcDNA3, and basic; Student's *t*-test). Moreover, the mutant HBx protein was able to remarkably increase the activities compared to the wild-type HBx protein (P<0.01 *vs* pCMV-X; Student's *t*-test; Figure 3).

Truncated HBx protein at COOH terminal upregulates expression of proteins related to cell proliferation To investigate the molecular mechanism, we examined the regulation of proteins, such as c-Myc and PCNA by the mutant HBx proteins. The Western blot analysis showed that the expression of c-Myc and PCNA were upregulated by the mutant HBx proteins and wild-type HBx protein (Figure 4A). Moreover, the expression levels of c-Myc and PCNA were upregulated in both L-O2-X-Sera cells and L-O2-XΔ127 cells than that in L-O2-X cells. We further confirmed this finding by applying Glyco BandScan software (PROZYME, San Leandro, CA, USA; Figure 4B).

Discussion

Previous studies showed that the mutation of the HBx gene plays a crucial role in the development of HBV-related HCC. In the present study, we found that the HBx gene showed heterogeneity, and both the wild-type and mutant HBx gene could be detected in the tumor tissues. HBx mutants, especially the integrated HBx gene, displayed the loss of different size of fragments at the carboxyl terminal[3,18]. Reports on HBx mutation vary depending on different geographic regions. It has been reported that nt382–389 (codons 128–130 aa) in HCC samples collected from Qidong, China[19] and nt93 (codon 31 aa) in HCC samples from Taiwan[20] of the HBx gene were found, which were the hot spots of mutation. Recently, Xu *et al* reported that human APOBEC3 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3) led to G-to-A mutation at positions 359 and 360 (TGG to TAA, TAG, or TGA) which generated a premature stop codon at position 120 amino acid in the HBx gene, resulting in the synthesis of a truncated HBx protein missing the last 35 amino acids. It caused a gain of function that enhanced the colony-forming ability and proliferative capacity of neoplastic cells[21].

In our present study, we found a natural HBx mutant from the sera (Figure 1A,1B) and HCC tissues (Figure 1C) of patients. According to the reports, 3 regions of the HBx protein, such as amino acids 46–52, 61–69, and 132–139, may be essential for the transactivation function of the HBx protein[22]. Here, we found that the mutant with deletion (128–154 amino acids) of the HBx protein exactly includes the regions of amino acids 132–139. It was also reported that the N terminal (amino acids 1–50) was important for transformation[23]. Here, we found that the mutant pattern of the HBx gene in HCC tissues was consistent with the ones in the sera, suggesting that the natural mutant of the HBx protein-truncated 27 amino acids at the COOH terminal may involve the development of HCC. According to the clinical examination of HBV antigens and antibodies to HBV antigens (Tables 1,2), 7 out of 8 patients with the HBx mutants were male, and all were positive for HBsAg and antibody to hepatitis B core antigen (anti-HBc) in the sera; 6 out 8 patients were positive for antibody to hepatitis B e antigen (HBeAg) in the sera. This suggests that the mutant may be related to the

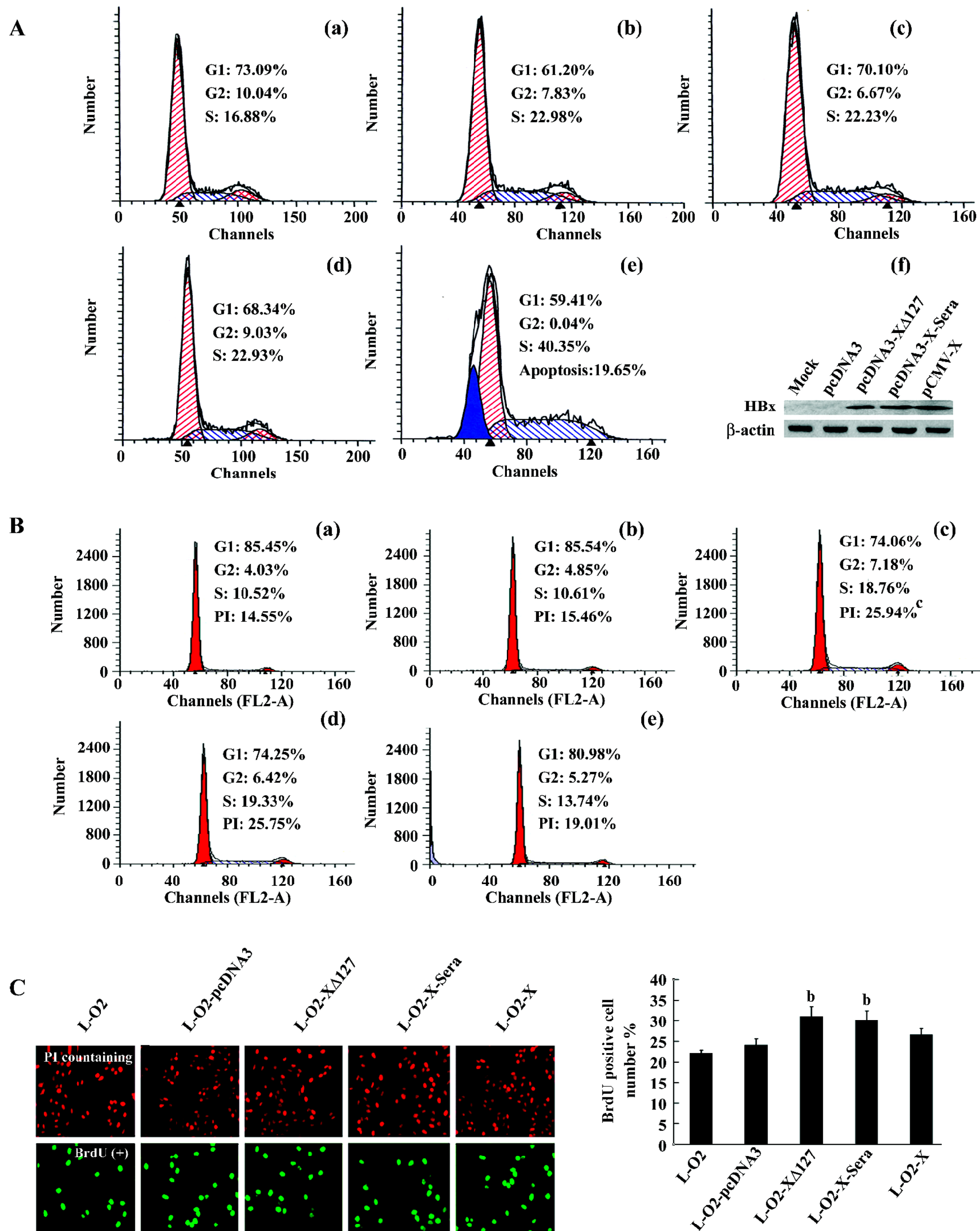

Figure 2. Effect of the mutant HBx protein on apoptosis and the proliferation of L-O2 cells. (A) mutant HBx protein lost the capability of inducing apoptosis in transient transfection by flow cytometry analysis. (a) L-O2 cells without transfection; (b) L-O2 cells transfected with empty control vector pcDNA3; (c) L-O2 cells transfected with plasmid carrying the artificially truncated HBx gene pcDNA3-XΔ127; (d) L-O2 cells transfected with plasmid carrying the natural mutant HBx gene pcDNA3-X-Sera; (e) L-O2 cells transfected with plasmid pCMV-X, which induced 19.65% apoptosis; (f) expression of the HBx protein was shown in the transfected L-O2 cells by Western blot analysis. (B) mutant HBx proteins promoted the proliferation of L-O2 cells in stable transfection. PI of cells was examined by flow cytometry analysis. (a) L-O2 cells; (b) L-O2-pcDNA3 cells; (c) L-O2-XΔ127 cells; (d) L-O2-X-Sera cells; (e) L-O2-X cells. (C) mutant HBx proteins promoted the proliferation of L-O2 cells by stable transfection, as examined by the BrdU incorporation assay. Propidium iodine staining positive cells was red in the nucleus, and green fluorescence showed the number of BrdU-positive cells. (D) histogram showed the positive rates of BrdU-positive cells. ${}^{b}P<0.05$, ${}^{c}P<0.01$ *vs* control or L-O2-X; Student's *t*-test.

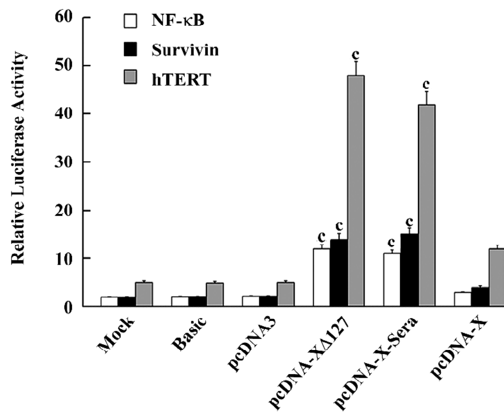

Figure 3. Mutant HBx protein promotes transcriptional activities of NF-κB, survivin, and hTERT. L-O2 cells were transiently transfected with the empty control vector pcDNA3, pcDNA3-XΔ127, pcDNA3-X-Sera, and pCMV-X. In each group, cells were cotransfected with the empty vector pGL3-Basic, pGL3-NF-κB, pGL3-survivin, and pGL3-hTERT, respectively. Luciferase activity of cells cotransfected with pcDNA3 and pGL3-Basic was set to 1, and the activities found in other transfections are shown relative to this. Data are from 3 independent experiments with standard errors shown as error bars. $^{c}P<0.01$ *vs* controls, such as mock, pcDNA3 empty vector, and pGL3-basic plasmid; Student's *t*-text.

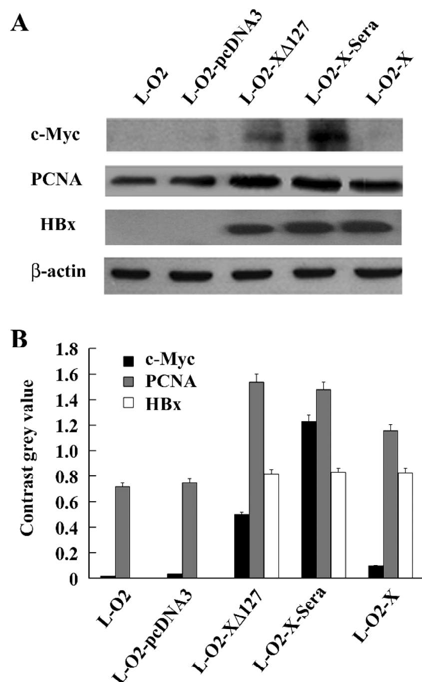

Figure 4. Mutant HBx protein upregulates proteins associated with cell proliferation. (A) c-Myc, PCNA, and HBx were examined by Western blot analysis in L-O2 cells and stably-transfected L-O2 cells. (B) bands were analyzed with the use of Glyco BandScan software.

active replication of HBV DNA. The clinical significance of the HBx mutant needs to be further investigated.

In order to demonstrate the biological activities of the mutant, we investigated the effect of the mutant on apoptosis and cell proliferation. Our data showed that wild-type HBx protein could remarkably induce apoptosis, but the mutant HBx protein failed to induce apoptosis (Figure 2A). HBx has a dual function in the stimulation of cell proliferation and induction of apoptosis by p53-dependent mechanisms. HBx can induce cell apoptosis in a p53-independent manner[3]. In addition, it was reported that HBx can downregulate p21 expression by suppressing p53 expression via protein–protein interaction[24], which might play a central role in cell malignant transformation. The distal COOH terminal region of HBx has been proven critical for binding to p53[6]. Therefore, in our study, the mutant HBx protein, which lost its capability to induce apoptosis, may be related to HBx–p53 interaction.

A large amount of evidences shows that full-length and the truncated HBx protein may play different roles in HBV-related HCC development. The HBx COOH terminal amino acids have been investigated to play a key role in regulating its transcriptional activity and controlling cell viability and proliferation[2]. In our experiments we found that L-O2-X-Sera and L-O2-XΔ127 cells grew remarkably faster than L-O2-X cells (Figure 2B–2D). Luciferase reporter gene assay (Figure 3) provided evidences that the HBx protein truncated at the COOH terminal is able to stimulate the promoter transcriptional activities of NF-κB, survivin, and hTERT in L-O2 cells, suggesting that the natural mutant has the potential to promote cell growth through the transcriptional activity of NF-κB, survivin, and hTERT. The Western blot analysis revealed that c-Myc and PCNA were upregulated by the mutant (Figure 4). Previous studies showed that HBx can accelerate tumor development induced by c-Myc. The coexpression of HBx and c-Myc transgenes accelerated HCC development in transgenic mice, and the cells with high c-Myc expression induced by HBx showed no alteration in p53 expression[25]. In addition, another study reported that the mutant HBx protein truncated at COOH terminal enhanced the transforming ability of ras and myc[2]. Therefore, we consider that the COOH terminal-truncated HBx protein is able to enhance cell proliferation through NF-κB and c-Myc, etc. However, the detailed molecular mechanisms involved in the switch of HBx function in terms of apoptosis, proliferation, and tumorigenesis need to be clarified in further studies.

In summary, we identified a natural mutant of the HBx gene from the sera and tissues of Chinese patients with

chronic liver diseases. Our findings provide an insight into the roles of COOH terminal amino acids of the HBx protein in the pathogenesis of HBV. Our data are significant, and provide theoretical and practical value for early diagnosis, prevention, and therapy of HCC.

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