

## Apoptosis and its pathway in X gene-transfected HepG<sub>2</sub> cells

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### Abstract

**AIM:** To investigate the effect of hepatitis B virus (HBV) X gene on apoptosis and expressions of apoptosis factors in X gene-transfected HepG<sub>2</sub> cells.

**METHODS:** The HBV X gene eukaryon expression vector *pcDNA<sub>3</sub>-X* was transiently transfected into HepG<sub>2</sub> cells by lipid-media transfection. Untransfected HepG<sub>2</sub> and HepG<sub>2</sub> transfected with *pcDNA<sub>3</sub>* were used as controls. Expression of HBx in HepG<sub>2</sub> was identified by RT-PCR. MTT and TUNEL were employed to measure proliferation and apoptosis of cells in three groups. Semi-quantified RT-PCR was used to evaluate the expression levels of Fas/FasL, Bax/Bcl-xL, and c-myc in each group.

**RESULTS:** HBV X gene was transfected into HepG<sub>2</sub> cells successfully. RT-PCR showed that HBx was only expressed in HepG<sub>2</sub>/*pcDNA<sub>3</sub>-X* cells, but not expressed in HepG<sub>2</sub> and HepG<sub>2</sub>/*pcDNA<sub>3</sub>* cells. Analyzed by MTT, cell proliferation capacity was obviously lower in HepG<sub>2</sub>/*pcDNA<sub>3</sub>-X* cells ( $0.08910 \pm 0.003164$ ) than in HepG<sub>2</sub> ( $0.14410 \pm 0.004927$ ) and HepG<sub>2</sub>/*pcDNA<sub>3</sub>* cells ( $0.12150 \pm 0.007159$ ) ( $P < 0.05$  and  $P < 0.01$ ). Analyzed by TUNEL, cell apoptosis was much more in HepG<sub>2</sub>/*pcDNA<sub>3</sub>-X* cells (980/2 000) than HepG<sub>2</sub> (420/2 000), HepG<sub>2</sub>/*pcDNA<sub>3</sub>* cells (520/2 000) ( $P < 0.05$  and  $P < 0.01$ ). Evaluated by semi-quantified RT-PCR, the expression level of Fas/FasL was significantly higher in HepG<sub>2</sub> cells transfected with HBx than in HepG<sub>2</sub> and HepG<sub>2</sub>/*pcDNA<sub>3</sub>* cells ( $P < 0.05$  and  $P < 0.01$ ). Bax/Bcl-xL expression level was also elevated in HepG<sub>2</sub>/*pcDNA<sub>3</sub>-X* cells ( $P < 0.05$  and  $P < 0.01$ ). Expression of c-myc was markedly higher in HepG<sub>2</sub>/*pcDNA<sub>3</sub>-X* cells than in HepG<sub>2</sub> and HepG<sub>2</sub>/*pcDNA<sub>3</sub>* cells ( $P < 0.05$  and  $P < 0.01$ ).

**CONCLUSION:** HBV X gene can impair cell proliferation capacity, improve cell apoptosis, and upregulate expression of apoptosis factors. The intervention of HBV X gene on the expression of apoptosis factors may be a possible mechanism responsible for the change in cell apoptosis and proliferation.

**Key words:** HBx; Transfect; HepG<sub>2</sub>; Apoptosis; Fas; FasL; Bax; Bcl-xL; c-myc

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### INTRODUCTION

Hepatitis B virus (HBV) is a small DNA virus with partial double-stranded DNA genome. HBV contains four opening reading frames (ORF) namely preS<sub>1</sub>/preS<sub>2</sub>/S, preC/C, P, and X. X gene is a unique ORF which is well conserved in different mammalian hepadnaviruses, its product consists of 154 amino acids with a molecular weight of 16.7 ku. Based on epidemical data, HBx is thought to be associated with HBV-related primary hepatocellular carcinoma (HCC), but the molecular basis for the oncogenic activity of HBx remains elusive. HBx is a multiple-functional protein and plays an essential role in viral pathogenesis. HBx can deregulate cell cycle check points, transactivate cells and viral genes, which involve in transcription regulation, single transduction pathway, cell cycle regulation, *etc.*<sup>[1]</sup> It has been shown that HBx can co-ordinate balance between proliferation and programmed cell death, and it is able to induce or block apoptosis. The deregulation of apoptosis is involved in a wide range of pathological processes, including development of HCC.

In the present study, we investigated the effect of HBx expression on apoptosis in human hepatoma cell line HepG<sub>2</sub>, and its effect on the expression level of apoptosis factors.

### MATERIALS AND METHODS

#### Materials

*PcDNA<sub>3</sub>* expression vector and HBV X gene eukaryon expression vector *pcDNA<sub>3</sub>-X* were previously constructed. Human hepatoma cell line HepG<sub>2</sub> was provided by Cell Bank of Chinese Academy of Sciences. Modified Eagle's medium (MEM) was bought from Hyclone Company, USA. Reverse transcription system, DNA purification system, and Transfast™ transfection reagent were obtained from Promega Biotech (USA). Total RNA isolation kit was purchased from Jingmei Biotech Company (Shanghai, China). PCR primers were synthesized by Shanghai Biotechnology Company. In-site cell apoptosis detection kit was provided by Roche Company.

#### Methods

**Cell culture and DNA transfection** HepG<sub>2</sub> cells were cultured in MEM supplemented with 10% heat-inactivated

fetal bovine serum, 100 IU/mL penicillin and 100 mg/mL streptomycin in a humidified incubator with 50 mL/L CO<sub>2</sub>. A total of 1.5×10<sup>6</sup> cells/mL were seeded into a 25 cm<sup>2</sup> cell plate before the experiment. When cells were grown to 80% confluence, *pcDNA<sub>3</sub>* or *pcDNA<sub>3</sub>-X* plasmid was transfected into HepG<sub>2</sub> cells by lipofection technique, which were named as HepG<sub>2</sub>/*pcDNA<sub>3</sub>* cells and HepG<sub>2</sub>/*pcDNA<sub>3</sub>-X* cells. A mixture containing 2 mL serum-free MEM (prewarmed to 37 °C), 5 µg plasmid DNA, 15 µL transfect reagent was added to a cell plate. After incubation for 24 h, 4 mL complete medium was added into cell plate and then incubated for another 48 h. HepG<sub>2</sub> cells, untransfected with any plasmid DNA, were used as controls.

**Detection of X gene expression by RT-PCR** Total RNA was extracted from HepG<sub>2</sub>, HepG<sub>2</sub>/*pcDNA<sub>3</sub>*, and HepG<sub>2</sub>/*pcDNA<sub>3</sub>-X* cells respectively, and reverse transcribed into cDNA. One microliter of RT product was used as template, PCR was carried out. The sequences of X gene primers were: 5'-ATGCAAGCTTATGGCTGCTAGGC-TGTACTG-3' and 5'-TGCGAATTCTTAGGCAGAGG-TGAAAAAGTTG-3'. The expected amplification fragment was 467 bp. PCR conditions were as follows: pre-denaturation at 95 °C for 5 min, 32 amplification cycles (denaturation at 94 °C for 35 s, annealing at 65 °C for 35 s, and extension at 72 °C for 1 min), and a final extension at 72 °C for 7 min. The PCR products were separated by electrophoresis on 1.5% agarose gel, and detected by ultraviolet radiography.

**Cell viability assay** Cell viability was assayed by MTT. HepG<sub>2</sub>, HepG<sub>2</sub>/*pcDNA<sub>3</sub>*, and HepG<sub>2</sub>/*pcDNA<sub>3</sub>-X* cells were planted into 96-well plates. Cells in logarithmic growth were used in experiments. One day before the experiment, complete medium was replaced by serum-free medium. During experiment, 75 µL MTT (5 mg/mL, containing in 0.01 mol/L PBS) was added into each well and incubated for 4 h. Then, the medium was replaced by DMSO (75 µL each well) and shaken gently until all crystals were dissolved. A<sub>492</sub> was detected to measure the proliferative capacity of each group.

**Cell apoptosis assay** Cell apoptosis was estimated by TUNEL staining. HepG<sub>2</sub>, HepG<sub>2</sub>/*pcDNA<sub>3</sub>*, and HepG<sub>2</sub>/*pcDNA<sub>3</sub>-X* cells were planted into 96-well plates. Cells at 80% confluence were fixed with 4% paraformaldehyde, and chilled in ice

bath for 2 min with permeabilization solution (0.1% Triton X-100 in 0.1% sodium nitrate). Then, 50 µL TUNEL mixture was added, incubated in a humidified chamber at 37 °C for 1 h. TUNEL mixture was removed, 50 µL Converter-AP was added and incubated for another 30 min. The cells were rinsed with PBS, counterstained with NBT/BCIP, and detected by optic microscopy.

**Effect of HBx transient transfection on apoptosis factor mRNA expression** Expressions of Fas/FasL, Bax/Bcl-xL, and c-myc gene were assayed by semi-quantitative RT-PCR. β-Actin was used as internal control. Total RNA was extracted respectively with RNA isolation kit, and reverse transcribed into cDNA. PCR was performed in a 50 µL reaction volume containing 5 µL 10× PCR buffer, 5 µL 2 mmol/L MgCl<sub>2</sub>, 1 µL 10 mmol/L dNTP, 1 µL 20 pmol/µL target gene sense and anti-sense primers, 0.5 µL 12.5 pmol/µL β-actin primer pair, 2 µL RT product, 1.5 U Taq DNA polymerase. The sequences of gene primers and amplification conditions are listed in Table 1. The initial denaturation was at 94 °C for 5 min. An additional extension step at 72 °C for 10 min was done finally. About 10 µL PCR products was separated by electrophoresis on 1.5% agarose gel, and detected by ultraviolet radiography. The densities of bands were analyzed by Bio imaging system, the ratio of target gene density to β-actin density was representative of the relative expression level of mRNA. The semi-quantitative detection was analyzed five times.

### Statistical analysis

All data were expressed as mean±SE. The significance for the difference between groups was assessed with SPSS 10.0 by one-way ANOVA. *P*<0.05 was considered statistically significant.

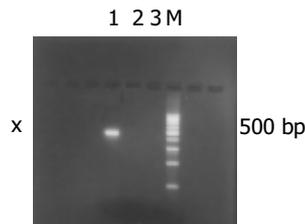
## RESULTS

### Expression of HBV X mRNA in HepG<sub>2</sub> cells

Expressions of HBx mRNA were detected by RT-PCR. The expected band between 400 and 500 bp was found in HepG<sub>2</sub>/*pcDNA<sub>3</sub>-X* cells, but not in HepG<sub>2</sub> and HepG<sub>2</sub>/*pcDNA<sub>3</sub>* cells (Figure 1).

**Table 1** Sequences of gene primers and amplification conditions

Target gene	Primer sequences	Amplification conditions	Product (base)
Fas	5'-TCA GTA CCG AGT TGG GGA AG-3' 5'-CAG GCC TTC CAA GTT CTG AG-3'	Denaturation at 94 °C for 45 s, annealing at 64 °C for 30 s, extension at 72 °C for 1 min, 35 cycles	207 bp
FasL	5'-GAT GAT GGA GGG GAA GAT GA-3' 5'-TGG AAA GAA TCC CAA AGT GC-3'	Denaturation at 94 °C for 45 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, 38 cycles	203 bp
Bax	5'-TTT GCT TCA GGG TTT CAT CC-3' 5'-CAG TTG AAG TTG CCG TCA GA-3'	Denaturation at 94 °C for 45 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, 30 cycles	246 bp
Bcl-xL	5'-GGC TGG GAT ACT TTT GTG GA-3' 5'-ATG TGG TGG AGC AGA GAA GG-3'	Denaturation at 94 °C for 45 s, annealing at 64 °C for 30 s, extension at 72 °C for 45 s, 25 cycles	198 bp
c-myc	5'-TTC GGG TAG TGG AAA ACC AG-3' 5'-CAG CAG CTC GAA TTT CTT CC-3'	Denaturation at 94 °C for 45 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, 30 cycles	203 bp
β-actin	5'-GGC ATC GTG ATG GAC TCC G-3' 5'-GCT GGA AGG TGG ACA GCG A-3'	Changed according to different target genes	607 bp



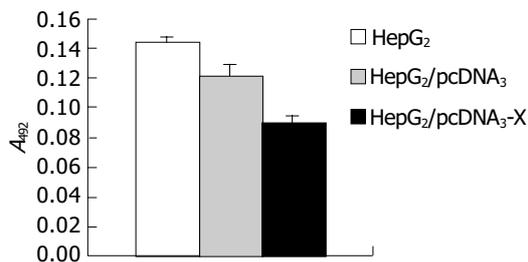
**Figure 1** Expression of HBV X mRNA in HepG<sub>2</sub> cells. M: 100-bp DNA ladder; lane 1: HepG<sub>2</sub>/pcDNA<sub>3</sub>-X cells; lane 2: HepG<sub>2</sub>/pcDNA<sub>3</sub> cells; lane 3: HepG<sub>2</sub> cells.

### Cell viability assay

Cell viability was assessed by MTT. All data are shown in Table 2.  $A_{492}$  of HepG<sub>2</sub>/pcDNA<sub>3</sub>-X cells obviously decreased compared to that in other groups ( $P < 0.05$  and  $P < 0.01$ ), indicating that transient expression of HBx impaired the proliferative capacity of HepG<sub>2</sub> cells (Figure 2).

**Table 2**  $A_{492}$  of HepG<sub>2</sub>, HepG<sub>2</sub>/pcDNA<sub>3</sub> and HepG<sub>2</sub>/pcDNA<sub>3</sub>-X cells (mean±SE)

Group	n	$A_{492}$
HepG <sub>2</sub>	10	0.14410±0.004927
HepG <sub>2</sub> /pcDNA <sub>3</sub>	10	0.12150±0.007159
HepG <sub>2</sub> /pcDNA <sub>3</sub> -X	10	0.08910±0.003164



**Figure 2** Effect of HBx on cell viability in HepG<sub>2</sub> cells.

### Cell apoptosis assay

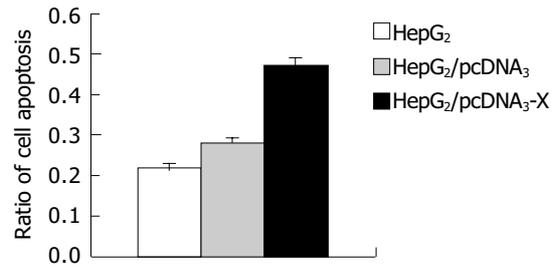
Apoptosis in the three groups was assessed with in-site cell death detecting kit (TUNEL). A total of 2 000 cells of each group were calculated. The number of apoptosis cells was 980 in HepG<sub>2</sub>/pcDNA<sub>3</sub>-X, 520 in HepG<sub>2</sub>/pcDNA<sub>3</sub> and 420 in HepG<sub>2</sub>. Cell apoptosis markedly increased in HepG<sub>2</sub>/pcDNA<sub>3</sub>-X (Table 3 and Figure 3), indicating that transient expression of HBx could promote apoptosis of HepG<sub>2</sub> cells (Figure 4).

**Table 3** Effect of HBx on cell apoptosis of HepG<sub>2</sub> cells (mean±SE)

Group	n	Apoptosis
HepG <sub>2</sub>	5	0.2200±0.1000
HepG <sub>2</sub> /pcDNA <sub>3</sub>	5	0.2800±0.1000
HepG <sub>2</sub> /pcDNA <sub>3</sub> -X	5	0.4750±0.015

### Effects of HBx transient transfection on apoptosis factors' mRNA expression

**Fas/FasL mRNA** mRNA level of Fas and FasL was



**Figure 3** Effect of HBx on apoptosis of HepG<sub>2</sub> cells.

elevated in HepG<sub>2</sub>/pcDNA<sub>3</sub>-X cells (Figures 5A and B, 6A and B), indicating that transient expression of HBx induced expression of Fas and FasL in HepG<sub>2</sub> cells.

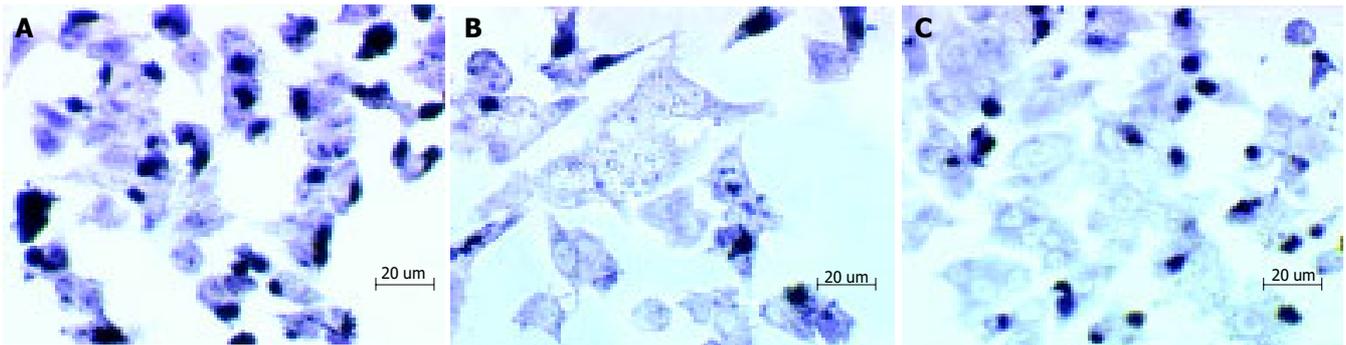
**Bcl-xL/Bax mRNA** mRNA level of Bcl-xL and Bax was enhanced in HepG<sub>2</sub>/pcDNA<sub>3</sub>-X cells (Figures 5C and D, 6C and D), indicating that transient expression of HBx induced expression of Bcl-xL or Bax in HepG<sub>2</sub> cells.

**C-myc mRNA** mRNA level of c-myc in HepG<sub>2</sub>/pcDNA<sub>3</sub>-X cells was the highest (Figures 5E and 6E), indicating that transient expression of HBx induced expression of c-myc in HepG<sub>2</sub> cells.

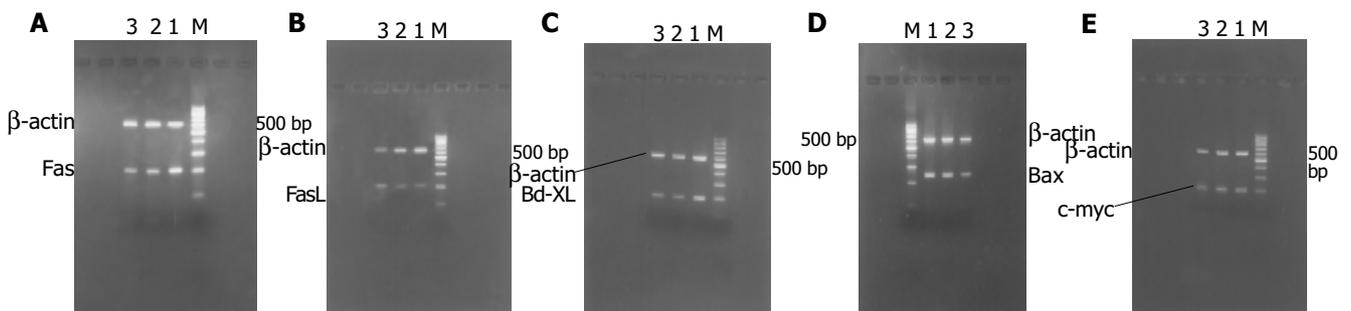
## DISCUSSION

In previous studies, it was found that HBx inhibits cell apoptosis in different ways. For example, HBx antagonizes TNF- $\alpha$ -induced apoptosis through activating PI3-kinase signaling pathway<sup>[2]</sup>, and inhibits apoptosis in p53-independent manner<sup>[3]</sup>. There is evidence that HBx activates NF- $\kappa$ B and induce it to translocate into nuclei, NF- $\kappa$ B acts as an inhibitor of cell apoptosis; HBx also downmodulates expression of Bid and blocks Bid-mediated cell apoptosis<sup>[4]</sup>, inactivates caspase-3 through inhibition of CCP32 enzyme, and blocks caspase pathway<sup>[5]</sup>. It is thought that anti-apoptosis function of HBx is an important mechanism in the development of HCC.

HBx can either inhibit or promote cell apoptosis in a dose-dependent manner. When HBx expresses at high level, it displays pro-apoptosis effect; whereas it inhibits apoptosis when expressing at physiological level. It was reported that moderate expression level of HBx can inhibit liver regeneration in HBx-expressing transgenic mice after partial hepatectomy<sup>[6]</sup>. HBx stimulates expression of FasL, which plays an important role in cell's escaping from immune surveillance by inducing apoptosis of T cell bearing Fas<sup>[7,8]</sup>. HBx boosts cell survival by abrogating Bcl-2-mediated cell protection<sup>[9,10]</sup>. It can also induce expression of myc protein in certain settings, myc sensitizes cells to be killed plus TNF- $\alpha$ <sup>[11]</sup>. HBx acts through the way that involves DDB2-independent nuclear function of DDB1<sup>[12]</sup>. Some researchers found that HBx can also localize in mitochondria, bind to voltage-dependent anion channel, which results in alteration of the mitochondrial transmembrane potential, promotes cytochrome C and apoptosis-inducing factors to release into cytosol and induces cell apoptosis<sup>[13]</sup>. In short, HBx has bi-directional function on cell apoptosis regulation. HBx expression levels, availability of survival *vs* apoptogenic factors, and stage of infection may profoundly influence



**Figure 4** Apoptosis in HepG<sub>2</sub>/pcDNA<sub>3</sub>-X (A), HepG<sub>2</sub>/pcDNA<sub>3</sub> (B), and HepG<sub>2</sub> (C) cells.



**Figure 5** RT-PCR results of mRNA expression of Fas (A), FasL (B), Bcl-xL (C), Bax (D), and c-myc (E) in HepG<sub>2</sub>. M: 100-bp DNA ladder; lane 1: HepG<sub>2</sub>

cells; lane 2: HepG<sub>2</sub>/pcDNA<sub>3</sub> cells; lane 3: HepG<sub>2</sub>/pcDNA<sub>3</sub>-X cells.

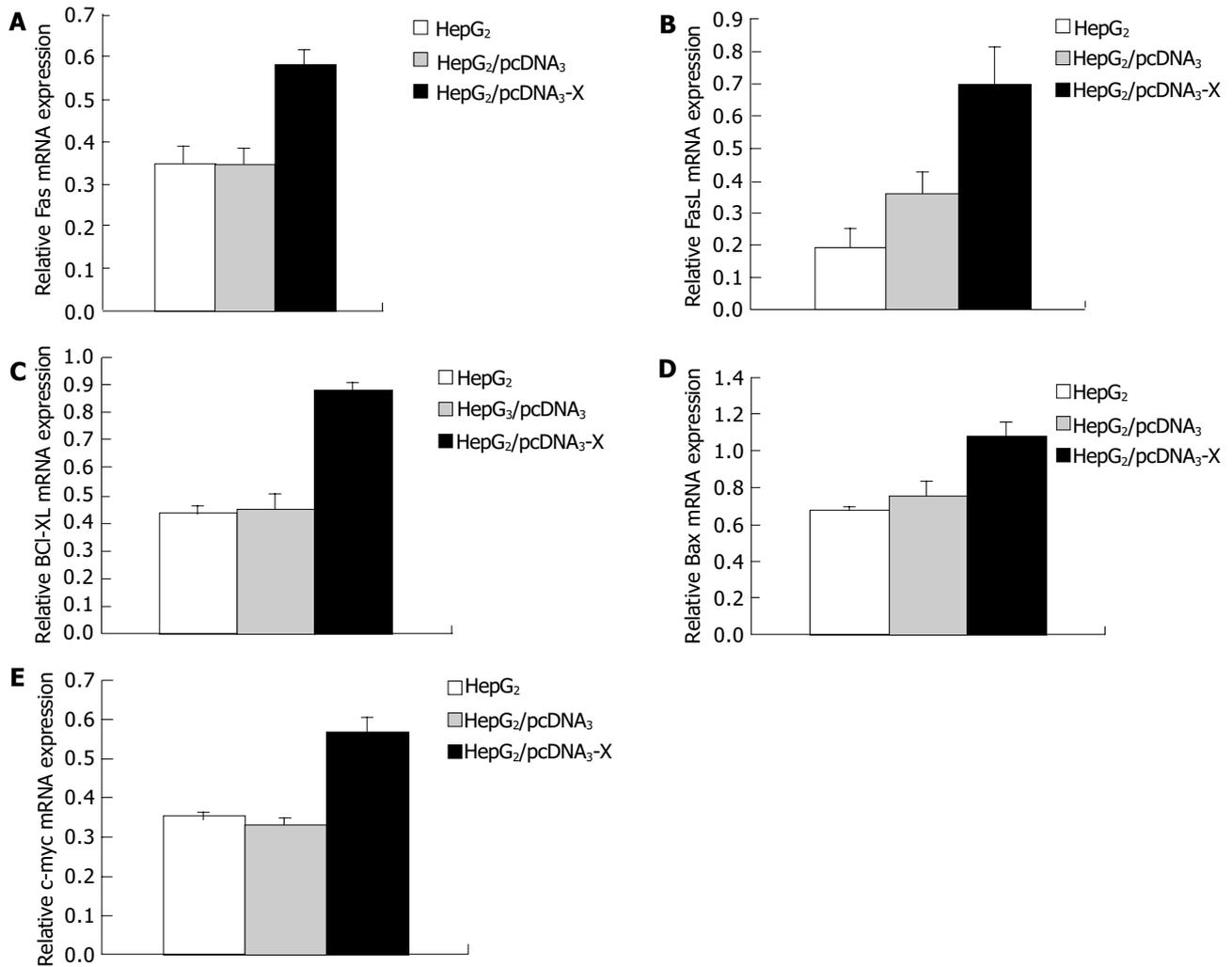
the fate of cells.

Higher organisms have several mechanisms to eliminate cells by apoptosis. One important role is the signaling pathway mediated by “death factors” including TNFR1, Fas, TNFR2, and their cognate ligand (TNF- $\alpha$ , FasL, and TRAIL) Fas (CD<sub>95</sub>). The first identified member of “death factors”, is a type I glycoprotein which expresses on cell surface. Crosslinking Fas by binding to the ligand FasL leads to conformational changes of Fas, which results in formation of death induced signal complex (DISC) followed by activation of caspase-8. Activated caspase-8 activates itself and other caspases that switch on apoptosis signal cascade<sup>[14,15]</sup>. It has been found that Fas and FasL express in hepatocytes and hepatoma cells. Since hepatocytes are highly sensitive to Fas/FasL-mediated apoptosis, Fas/FasL pathway plays an essential role in liver lesion and eliminating virus. In our research, HBx elevated expression of Fas and FasL in HepG<sub>2</sub> cells. Although the precise mechanism remains unclear, HBx can activate FasL promoter through binding site for Egr and enhance Egr binding to the co-activator cAMP-response element-binding protein, and induce pro-inflammatory cytokines at transcriptional level such as IL-18 which can amplify the expression of FasL<sup>[16]</sup>. c-FLIP, a key regulator of the DISC, inhibits the Fas/FasL-mediated death pathway in tumors. HBx abrogates the apoptosis-inhibiting function of c-FLIP and renders cells hypersensitive towards the TNF- $\alpha$  apoptotic signal even below the threshold concentration<sup>[17,18]</sup>.

Members of Bcl-2 family are also involved in apoptosis regulation. Members of this family are divided into three subgroups. One group is composed of anti-apoptosis proteins

such as Bcl-2, Bcl-xL, with four Bcl-2 homology domains (BH1, BH2, BH3, and BH4). Another group consists of pro-apoptosis proteins such as Bax, Bak, with BH1, BH2, BH3 domains. The last group includes pro-proteins such as Bid, Bik, with only BH3 domain<sup>[4,19]</sup>. As Bcl-2 family members reside in upstream of irreversible cell damage, they play a pivotal role in deciding whether cells die or live. Indeed, the ratio between pro- and anti-apoptosis molecules determines, in part, the susceptibility of cells to death signal<sup>[20]</sup>. It was reported recently that the anti-apoptosis members lose their ability to inhibit release of pro-apoptosis factors (such as cytochrome C) and trigger apoptosis if they interact with activated pro-apoptosis members<sup>[21]</sup>. Our data demonstrate that HBx upregulates either pro-apoptosis subset Bax or anti-apoptosis subset Bcl-xL. MTT and TUNEL displayed that apoptosis of HepG<sub>2</sub> cells transfected with HBx was enhanced while cell viability was impaired. We postulated that though HBx can upregulate expression of Bax and Bcl-xL, it may promote expression of Bax ever more than Bcl-xL, thus resulting in the predominance of pro-apoptosis protein in the ratio between pro- and anti-apoptosis subsets, then cell apoptosis. On the other hand, interacting with activated Bax, Bcl-xL may lose its anti-apoptosis function and trigger cell death.

C-myc belongs to cell oncogene. HBx accelerates development of primary liver tumors by co-operating with c-myc<sup>[22-25]</sup>. It was reported that myc can sensitize cells to apoptosis by about two folds in certain conditions such as exposure to TNF- $\alpha$  or other apoptosis factors. In our study, HBx promoted expression of c-myc. Overexpression of c-myc is essential for acute sensitization of cells to be killed



**Figure 6** Relative mRNA expression levels of Fas (A), FasL (B), Bcl-XI (C),

Bax (D), and c-myc (E) in HepG<sub>2</sub> cells.

by HBx plus TNF- $\alpha$ <sup>[11,26-28]</sup>, and may enhance cell apoptosis. This result agrees with the data of MTT and TUNEL in our study.

In hepatocarcinogenesis, preneoplastic, and neoplastic cells display an increased ratio of apoptosis as well as enhanced cell proliferation<sup>[30]</sup>. It is believed that the anti-apoptosis function of HBx is the major determinant factor for development of HCC. The pro-apoptosis function of HBx, however, is also thought to contribute to hepatocarcinogenesis. Firstly, HBx-induced cell apoptosis results in releasing of hepatocyte growth factor that enhances regeneration of liver cells and accumulation of genetic mutation, thus paving the way for cell malignant transformation<sup>[29-31]</sup>. Secondly, increased apoptosis increase the opportunity of mutation, leading cells to grow out of control and develop to HCC<sup>[28,32]</sup>. Thirdly, HBx induces expression of FasL in liver cells which can attack T cell bearing Fas and lead to impair of immune defense, which is beneficial for cell bearing HBx to escape from immune detection<sup>[33]</sup>. Finally, although the accurate mechanism of apoptosis induced by HBx has not been elucidated completely, it may facilitate propagation of viral infection by permitting efficient particle release from cells while minimizing the antiviral inflammation response<sup>[14]</sup>.

Further study should be focused on protein level. Besides, apoptosis mechanic on stably transfected HBx gene in HepG<sub>2</sub> cells is another pivot.

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