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# Involvement of extracellular signal-regulated kinase/mitogen-activated protein kinase pathway in multidrug resistance induced by HBx in hepatoma cell line

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## Abstract

**AIM:** To investigate the molecular mechanism of the influence of HBx protein on multidrug resistance associated genes: multidrug resistance 1 (MDR-1), multidrug related protein (MRP-1), lung resistance related protein (LRP) in hepatoma cells and the potential role of extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway in this process.

**METHODS:** A cell model stably expressing the HBx protein was established by liposome-mediated transfection of HBx gene into HepG2 cell line. The expression of multidrug resistance associated genes and proteins was detected by RT-PCR and Western blot. AnnexinV-FITC/PI assay was used to confirm the multidrug resistance (MDR) phenotype of transfected cells by fluorescence cytometry (FACS). The ERK/MAPK pathway activation was measured by Western blot through comparing the ratio of phosphorylation of ERK/MAPK to total ERK/MAPK protein. After treated with the ERK/MAPK pathway inhibitor U0126, the HBx-expressing cells were harvested. Then RT-PCR, Western blot and FACS were used to analyze the alterations in the expression of multidrug resistance associated genes and the MDR phenotype after exposure.

**RESULTS:** Compared with the control group, the transfected cells showed a higher expression of MDR associated genes and proteins. Marked elevations in MDR-1 (64.3%), MRP-1 (87.5%) and LRP (90.8%) were observed in the transfected cells ( $P < 0.05$ ). RT-PCR revealed that the over-expression of MDR associated proteins was due to amplification of such genes (MDR1 2.9 fold, MRP1 1.67 fold, LRP1.95 fold). Furthermore, we found that the ERK/MAPK activity was remarkably high in the HBx-expressing cells. The activation of ERK/MAPK, as measured by the ratio of phosphorylated ERK bands normalized to the total ERK bands, was increased by 2.3-fold in HBx-transfected cells compared with cells transfected with the empty vector. After treated with the ERK/MAPK pathway inhibitor, the level of MDR associated genes and proteins in the transfected cells decreased to some extent. Compared with controls, a significant decrease in MDR-1 mRNA (53.3%), MRP-1 mRNA (59.7%) as well

as LRP mRNA (56.4%) was observed in the U0126 treated transfected cells after 12 h. Western blot also demonstrated that the protein expression of these MDR associated genes slightly reduced after treated with U0126 for 12 h (MDR-1 40.1%, MRP-1 29.4%, LRP35.7%). This change was accompanied with the rise of cell apoptosis ratio confirmed by Annexin V-PI detection. The apoptosis index of U0126-treated cells increased by 1.28 fold, compared with that of transfected cells. Obviously, the MDR phenotype of these cells was obviously related with increased activities of the ERK/MAPK pathway.

**CONCLUSION:** HBx protein might be one of the causes for the occurrence of MDR in HCC, and ERK/MAPK pathway might be involved in this change.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers in China and approximately 90% of HCC cases in this region are associated with hepatitis B virus (HBV) infection<sup>[1,2]</sup>. The mechanism underlying the development of HCC is still unclear. X gene, a unique open reading frame of HBV, codes for a 16.5 kd protein (X protein, HBx)<sup>[3]</sup>. There is evidence that HBx is an essential viral protein with pleiotropic activity that might act directly or indirectly on development of hepatocellular carcinoma during chronic hepadnavirus infection<sup>[4,5]</sup>. Over-expression of HBx induces transformation of some cell types<sup>[6]</sup>. Additionally, it has been reported that HBx is involved in the arrest of cell cycle progression and the modulation of apoptosis<sup>[7,8]</sup>. HBx protein also interacts with the tumor suppressor *p53*, resulting in a loss of its function<sup>[9]</sup>. Due to its cytosolic localization, HBx has been shown to participate in a wide range of cellular signal transduction cascades, including Ras-Raf-mitogen-activated protein kinase, *c-Jun* N-terminal kinase, NF- $\kappa$ B and Janus kinase (JAK) -signal transducer and activator transcription factor (STAT) pathway<sup>[10-12]</sup>. It is well-known that tumor cells may avoid the death fate because apoptotic signals generated by the chemotherapeutic agents fail to activate the necessary pathways required to initiate apoptosis. Therefore, such survival signal pathways whose activation has been shown to play an anti-apoptotic role after treatment with various chemotherapeutic drugs have become the focus of recent researches. The mitogen-activated protein kinase (MAPK) family has emerged as one of such important membrane-to-nucleus signaling mechanisms of HBx, functioning as a mediator of cellular responses to a variety of cellular stimuli<sup>[13]</sup>. The MAPK family has been classified into 3 distinct subfamilies: the extracellular signal-regulated protein

kinases (ERKs) including ERK1 and ERK2, the stress-activated *c-Jun* N-terminal protein kinase (JNKs) and *p38* kinase<sup>[13]</sup>. Resistance to a broad spectrum of chemotherapeutic agents in cancer cells is called MDR<sup>[15]</sup>. The MDR phenotype is associated with the increased drug efflux from cells that is mediated by an energy-dependent mechanism and the blockade of the apoptosis-inducing pathway, the latter is the highlight of research nowadays. The results of experiments aiming at exploring the role of HBx on apoptosis pathways, however, are controversial and thence disappointed<sup>[15,16]</sup>. So we became more interested in determining whether these signal pathways were involved in the development of MDR, and investigated the potential role of MAPK pathways in multidrug resistance induced by HBx in hepatoma cells.

## MATERIALS AND METHODS

### Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), lipofectamine 2 000 and G418 were purchased from Invitrogen Inc. ERK/MAPK pathway inhibitor U0126, was purchased from Alexis Inc. Anti-phospho-ERK<sub>1</sub>/ERK<sub>2</sub> rabbit polyclonal antibody was purchased from RD systems. Anti-ERK<sub>1</sub>/ERK<sub>2</sub> rabbit polyclonal antibody was purchased from Biovision Inc. Polyclonal antibody to multidrug related protein (MRP-1), was from Alexis Inc. Lung resistance-related protein (LRP) mouse antibody, multidrug resistance 1 (MDR-1) mouse antibody and pan actin antibody-5 were provided by Neomarker Inc. Dimethyl sulfoxide (DMSO) was purchased from Sigma.

### Cell lines and cell culture

Human hepatocellular carcinoma cell line, HepG2 (GDC024) was purchased from the China Center for Type Culture Collection (Wuhan University, Hubei). Cells were maintained in DMEM supplemented with 100 mL/L heat-inactivated FBS at 37 °C in a humid atmosphere containing 50 mL/L CO<sub>2</sub>.

### Plasmids and stable transfection

The expression vector pcDNA3-HBx (a kindly gift from Lian-Rui Zhao, PhD; Thomas Jefferson University, USA) was constructed by inserting HBx DNA fragments between the *Hind*III and *Kpn*I cloning sites of the pcDNA3 vector. After amplification in *DH-5α*, we used the EZNA plasmid maxiprep kit (OMEGA, USA) to purify the plasmids. HepG2 cells at 70-80% confluence were transfected with plasmids, using lipofectamine 2 000 (Life Technologies, Inc.). Plasmid transfections were performed according to protocols supplied with the reagents. At 48 h post-transfection, cells were split at a 10:1 ratio and cultured in the presence of 800 g/L G418. After 21 d in selective medium, individual G418-resistant colonies were isolated. Expression of HBx in various cell lines was verified by RT-PCR and Western Blot. The control cell line (HepG2/pcDNA) was generated from cells transfected with the vector alone and selected using G418. After isolation of resistant clones, the concentration of G418 was changed to 400 µg/mL.

### Reverse transcriptase-PCR

Total cellular RNA of the stably transfected HepG2 cells was extracted with Trizol (Life Technologies, Grand Island, NY) according to the manufacturer's instructions and reverse transcribed into cDNA with 2 units of Moloney murine leukemia virus reverse transcriptase (MMLV-RT; MBI Fermentas Inc., Amherst, New York, USA), 4 µL of 10 mmol/L dNTPs (Fermentas, Inc.), 2 µL of oligo (dT), 10 µL of 5X reaction buffer (Fermentas Inc.), 2 µL of ribonuclease inhibitor and 26 µL of deionized water for 90 min at 37 °C. PCR was performed with 100 ng of cDNA in a 50 mL reaction volume containing 20 mmol/L Tris, pH 8.0, 50 mmol/L KCl, 2 mmol/L MgCl<sub>2</sub>, 10 mmol/L of each dNTP, 20 pmol of each primer, and 1.25 units of *Taq* DNA

polymerase (Fermentas, Inc.). The profile was 94 °C for 5 min before 25 cycles at 94 °C for 45 s, at Ta for 45 s, at 72 °C for 55 s, and a final extension at 72 °C for 10 min. All amplification products were separated on agarose gels and visualized by ethidium bromide staining under *uv* transillumination.

The primers used for semi-quantitative PCR were: sense 5'-TCT CCG TCT GCC GTT CCA -3' and antisense 5'-TCG GTC GTT GAC ATT GCT G-3' for HBx; sense 5'-TGA AGG ACT TCG TGT CAG CC-3 and antisense 5'-GTCC CATGAT GGT GTT GAG CC-3' for MRP-1; sense 5'-CAT TGG TGT GGT GAG TCA GG-3' and antisense 5'-CTC TCT CTC CAA CCA GGG TG-3' for MDR-1; sense 5'-TAA GGG CTT CCA GCA CCA AC-3' and antisense 5'-GGA GTT CTC GCT TCT CGT CC -3' for LRP and sense 5'-GTG CGT GAC ATT AAG GAG-3' and antisense 5'-CTA AGT CAT AGT CCG CCT-3' for β-actin.

### Protein preparation and Western blot analyses

Stably transfected HepG2 cells were rinsed with 0.01 mol/L PBS, and then lysed in a lysis buffer containing 50 mmol/L Tris-HCL (pH 8.5), 150 mmol/L NaCl, 0.2 g/L NaN<sub>3</sub>, 0.1 g/L SDS, 100 µg/mL PMSF, 1 µg/mL aprotinin, 10 mL/L NP-40, 5 g/L sodium deoxycholate. The products were centrifuged at 14 000 g for 15 min to remove the cellular debris. Protein concentrations were determined by the Bradford methods. A total of 50-100 µg of protein was separated by electrophoresis using 60 g/L or 100 g/L sodium dodecyl sulfate-polyacrylamide gels (according to the molecular mass of target protein), transferred to a immunoblot NC membrane, blocked with degreased milk for 12 h and hybridized with polyclonal antibodies for MRP-1, LRP, MDR-1, p-ERK and ERK (each diluted to 1:1000) at 4 °C overnight. The membranes were then washed 4 times for 15 min incubation with TBST (10 mmol/L Tris-Cl, pH 7.4, 150 mmol/L NaCl, 10 mL/L Tween 20 and hybridized with peroxidase (HRP) -conjugated secondary antibodies corresponding to each primary antibody. Protein banding specifically to the antibodies was visualized by ECL-associated fluorography (Pierce Inc.).

### Cell viability and apoptosis analysis by fluorescence cytometry (FACS)

Cells were plated and grown overnight until they reach 80% confluence, then the cells were treated with 20 µg/mL 5-Fu. Subsequently, detached cells in the medium were collected, and the remaining adherent cells were released by trypsinization. The cells were washed with phosphate-buffered saline (PBS) and resuspended in 250 µL binding buffer (annexinV-FITC kit; BD) containing 5 µL of annexin V-FITC stock and 10 µL of 20 µg/mL propidium iodide (PI) for the determination of phosphatidylserine (PS) exposure on the outer plasma membrane. After incubated for 10 min at room temperature in a light protected area, the samples were analyzed by a Becton Dickinson fluorescence cytometer using CellQuest software. We could discriminate intact cells (annexin<sup>-</sup>/PI<sup>-</sup>) from apoptotic cells (annexin<sup>+</sup>/PI<sup>+</sup>) and necrotic cells (annexin<sup>+</sup>/PI<sup>+</sup>) after treatment with 5-Fu.

### Statistical analyses

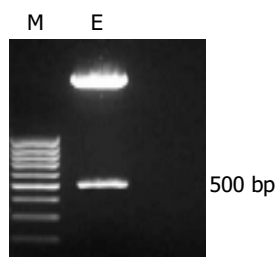
The results were expressed as mean±SE. Student's *t* test was used to compare data. *P*<0.05 was considered statistically significant. All data were processed by SPSS10.0.

## RESULTS

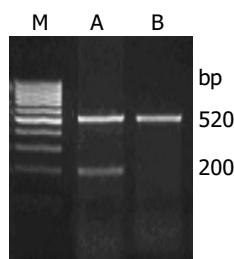
### Isolation and identification of HBx-expressing cells

As shown in Figure 1, the restriction enzyme digestions demonstrated that the digestion products had exactly the expected size (about 500 bp). We also requested Shanghai Bioasia Biotechnology Inc to sequence the whole plasmid kindly gifted by Lian-Rui Zhao (Ph.D), and the result of sequencing was consistent with the sequence of V0866 in GeneBank.

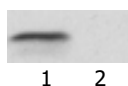
After stable transfection, the cells of different groups were confirmed for the expression of HBx by using PCR and immunoprecipitation. The identification of the successful transfection at mRNA and protein levels is shown in Figure 2 and Figure 3, respectively. The expected bands could be seen in the HBx-expressing cells and the groups treated with ERK/MAPK signal pathway inhibitors. There was no detectable band in the control group. These results indicated that HBx was successfully transferred and expressed in HepG2 cells.



**Figure 1** Restriction enzyme digestions of the plasmids. Lane M: markers (the brightest band of the marker presents the 500 bp); lane E: one of the split bands emerged at the exact position of 500 bp, and the other was the empty pcDNA3.0 vector as expected.



**Figure 2** Confirmation of the successful transfection of HBx into HepG2 cells by RT-PCR. Lane M: markers (the brightest band of the marker presents the 500 bp); lane A: transfected cells; lane B: transfected cells treated with U0126; lane C: the control.



**Figure 3** Western blot analysis of HBx protein in samples isolated from HepG2 cells with or without HBx transfection. Lane 1: the sample transfected with HBx; lane 2: the sample transfected with vector only.

#### Verification of MDR phenotype in HBx-expressing cells

To evaluate the effect of HBx introduction on the chemosensitivity of HepG2 cells, we initially used a FACS analysis of cells stained with PI and annexinV-FITC assay to compare the survival and apoptosis ratio of HepG2 cells transfected with empty pcDNA3.0 with HBx-transfected cells, i.e., HepG2/pcDNA and HepG2/pcDNA-HBx, after their exposure to 5-Fu (20 µg/mL) for 12 h and then cultured in normal medium for 6 h. Apoptosis index was quantified by a Becton Dickinson fluorescence cytometer. The apoptosis ratio in transfected cells ( $9.83 \pm 1.5\%$ ) analyzed by CellQuest software after administration of 5-Fu was significantly lower than that of the control ( $17.79 \pm 1.7\%$ ) (Figure 4). MTT assay also demonstrated that the inhibitory concentration 50% ( $IC_{50}$ ) of adriamycin in the transfected cells was higher than that of the control (data not shown). The multidrug resistance transporter MDR-1, MRP-1, and LRP were associated with multidrug resistance. Western blot analysis was performed to examine whether the resistance to 5-Fu-induced apoptosis in HBx-expressing cells was due to the alterations of such proteins. Marked elevations in

MDR-1 protein (64.3%), MRP-1 (87.5%) and LRP (90.8%) proteins were observed in the transfected cells (Figure 5 and Tables 1-2), which were well correlated with the degree of resistance. Furthermore, semi-quantitative RT-PCR revealed that over-expression of MDR-associated proteins was due to amplification of such genes (1.9-fold MDR-1, 0.67-fold MRP-1, 0.95-fold LRP) (Figure 6). Taken together, these data suggested that reintroduction of HBx contributed to the development of MDR phenotype.

**Table 1** Protein level of MDR associated genes (mean $\pm$ SD)

Group	MDR-1	MRP-1	LRP
transfected	$0.23 \pm 0.04$	$0.15 \pm 0.03$	$0.17 \pm 0.03$
control	$0.14 \pm 0.03$	$0.08 \pm 0.03$	$0.09 \pm 0.02$
P	0.036	0.046	0.018

**Table 2** mRNA level of MDR associated genes (mean $\pm$ SD)

Group	MDR-1	MRP-1	LRP
Transfected	$0.29 \pm 0.06$	$0.62 \pm 0.11$	$0.82 \pm 0.15$
Control	$0.10 \pm 0.03$	$0.37 \pm 0.09$	$0.42 \pm 0.07$
P	0.038	0.012	0.014

#### ERK/MAPK pathway levels and activities in transfected cells

To assess the role of ERK/MAPK in HBx-induced activation of MDR1 expression, we measured the phosphorylation of ERK/MAPK in cells transfected with HBx. As shown in Figure 6, the phosphorylation of ERK/MAPK as measured by the ratio of the phosphorylated ERK bands (Figure 7A) normalized to the total ERK band (Figure 7B), was increased by 2.3-fold in HBx-transfected cells compared with control cells. These results suggested that the MDR phenotype induced by HBx was accompanied with the activation of ERK/MAPK pathways.

#### Effect of ERK/MAPK pathway inhibitors on MDR of HBx-expressing hepatoma cells

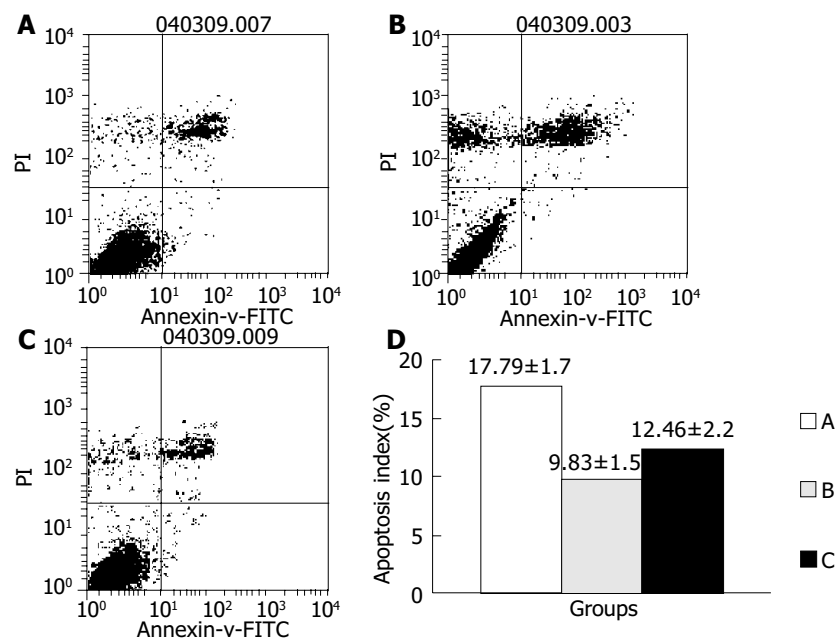
Based on the constitutive activation of ERK/MAPK, we determined whether U0126, a specific ERK inhibitor, could give rise to alterations in the expression of MDR associated genes and increase the transfected cell sensitivity to the cytotoxicity of 5-Fu. Treatment of transfected cells with U0126 decreased the levels of MDR-1 mRNA, as well as LRP and MRP-1 mRNA (Tables 3-4). Compared with controls, a significant decrease in MDR-1 mRNA (53.3%), MRP-1 mRNA (59.7%) as well as LRP mRNA (56.4%) was observed in U0126 treated transfected cells after 12 h. Western blot also demonstrated that the protein expression of these MDR associated genes was slightly reduced after treated with U0126 for 12 h (MDR-1 40.1%, MRP-1 29.4%, LRP 35.7%). The apoptosis index of U0126-treated cells increased by 1.28 fold, compared with that of transfected cells. All these observations proved that U0126 could impair the MDR phenotype induced by HBx and sensitize cells to the toxicity of 5-Fu.

**Table 3** Changes of MDR related genes after treated with U0126 (mean $\pm$ SD)

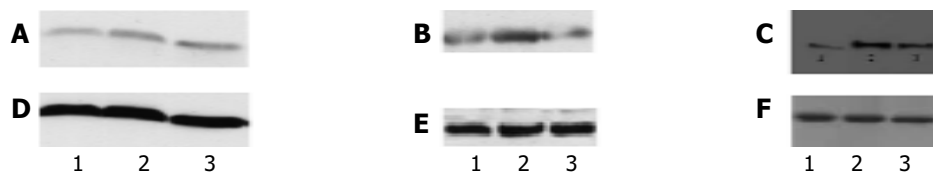
Group	MDR-1	MRP-1	LRP
Transfected	$0.15 \pm 0.03$	$0.62 \pm 0.11$	$0.78 \pm 0.13$
Treated with U0126	$0.08 \pm 0.02$	$0.37 \pm 0.09$	$0.44 \pm 0.09$
P	0.028	0.040	0.020

**Table 4** Changes of MDR related proteins after treated with U0126 (mean $\pm$ SD)

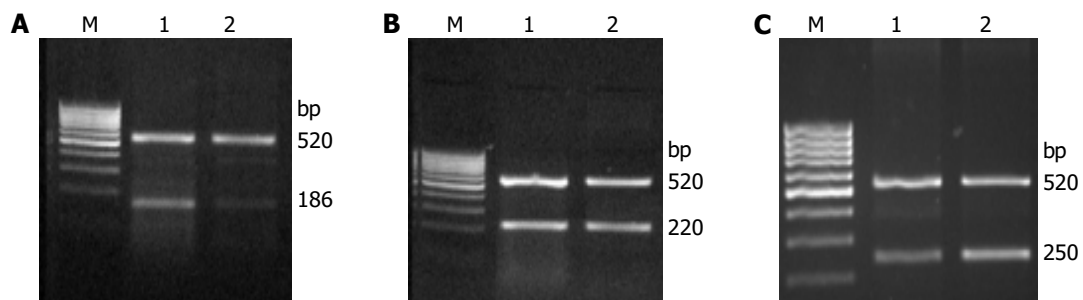
Group	MDR-1	MRP-1	LRP
Transfected	$0.22 \pm 0.04$	$0.17 \pm 0.02$	$0.14 \pm 0.02$
Treated with U0126	$0.13 \pm 0.04$	$0.12 \pm 0.02$	$0.09 \pm 0.02$
P	0.04	0.038	0.04



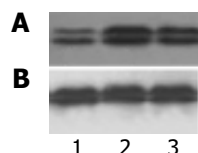
**Figure 4** Apoptosis index of three different groups of cells after treatment with 5-Fu. A: HepG2/pcDNA3 cells; B: HepG2/pcDNA3-HBx cells; C: U0126 pretreated transfected cells. D: Apoptotic index quantitated by FACS.



**Figure 5** Protein levels of MDR associated genes in different groups of cells. Lane1: HepG2/pcDNA3 cells; lane2: HepG2/pcDNA3-HBx cells; lane3: transfected cells treated with U0126. The primary antibody for A, B, C, D was  $\beta$ -actin, MDR-1, MRP-1 and LRP respectively.



**Figure 6** RT-PCR analysis of the expression of MDR associated genes in transfected and control cells. Lane 1: HBx-expressing cells; lane 2: control cells; lane M: markers. A: Specific primers to amplify MDR-1 gene; B: Specific primers to amplify MRP-1 gene; C: Specific primers to amplify LRP gene.

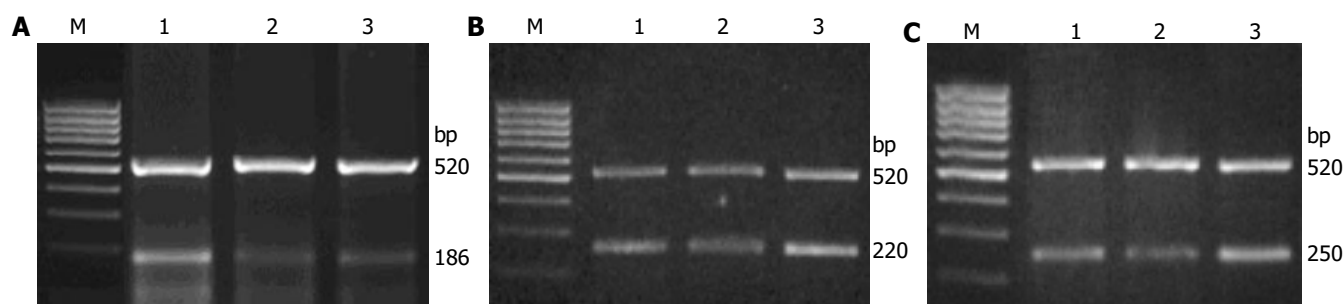


**Figure 7** Increased phosphorylation of ERK induced by HBx in hepatoma cells analyzed by Western blot. Lane 1: transfected cells after treated with U0126; lane 2: HBx-expressing cells; lane 3: control cells. A: Specific antibody for phosphorylated-ERK; B: Specific antibody for total ERK.

## DISCUSSION

MDR was the leading cause of failure to comprehensive therapy of HCC<sup>[17]</sup>. Although the detailed molecular mechanisms of MDR remain to be explored, the disorder of anti-apoptotic and pro-

apoptotic signal pathways and the over-expression of certain efflux proteins are generally believed to be due to MDR phenotype<sup>[18]</sup>. HBV infection is known as one of the most important risk factors in HCC development. Among the proteins coded by HBV genome, HBx has been proved to play a central role in virus duplication and carcinogenesis<sup>[2]</sup>. According to previous study, HBx could participate in several pathological processes of the generation and growth of HCC<sup>[2,4]</sup>. Apart from interfering with the suppressing function of *p53*, HBx could act as a multi-functional transcriptional transactivator to stimulate some important signal pathways. The effects of HBx on such signal pathways as ERK/MAPK, JNK, NF- $\kappa$ B and Src have been well-documented, especially in the aspect of apoptotic signals<sup>[16,19]</sup>. However, the pathological implications of these complex interactions in the evolvement of HCC malignant phenotypes have not been fully understood.



**Figure 8** RT-PCR analysis of the expression of MDR associated genes in HBx-expressing cells in response to treatment of U0126. Lane 1: HBx-expressing cells; lane 2: control cells; lane 3: transfected cells treated with U0126; lane M: marker. A: Specific primers to amplify MDR-1 gene; B: Specific primers to amplify MRP-1 gene; C: Specific primers to amplify LRP gene.

In this study, we investigated the influence of HBx protein on MDR associated proteins and the potential role of ERK/MAPK pathways in this process. Our data provided the evidence that HBx up-regulated the expression of MDR associated genes. Similar changes were witnessed in protein level. Our results indicated that HBx protein was a survival factor that could protect transfected cells from death induced by anti-tumor drugs in a constitutive expression system. Subsequent studies showed that HBx protein contributed to the MDR phenotype which was confirmed by apoptosis detection through FCAS. Furthermore, we demonstrated that the ERK/MAPK pathway worked as a downstream effector of HBx, mediating the MDR phenotype in the ERK/MAPK signal transduction pathway which was activated in HBx-expressing cells. To further confirm our previous hypothesis that the MAPK signal pathway played an important role in the MDR phenotype induced by HBx protein, we examined whether MAPK inhibited by U0126 (20  $\mu\text{mol/L}$ , 12 h) could alter the phenotype of MDR. The experimental results showed that P-glycoprotein (product of the *MDR-1* gene) mediated MDR of transfected cells is reduced by the treatment of such inhibitors, which supports corroborated our presumption.

Among the myriads of intracellular signaling molecules regulated by HBx, ERK/MAPK plays an important role in regulating several cellular processes, such as proliferation and differentiation<sup>[20]</sup>. Several studies suggested that ERK/MAPK was involved in the acquired resistance of cancer cells to cytostatic drugs<sup>[22,23]</sup>. Kisucka *et al.* reported that the effect of PD98059 and U0126 on vincristine resistance of L1210/VCR cells, suggesting that both substances were reversing agents of P-glycoprotein-mediated MDR, and the role of ERK-mediated phosphorylation cascades in the occurrence of MDR should be considered. Dent *et al.*<sup>[25]</sup> also demonstrated that the activation of the ERK/MAPK pathway played a critical role in leukemia cell survival after treatment with chemotherapeutic drugs and ionizing radiation. As shown in our experiment, the up-regulation of P-glycoprotein expression might be one of the mechanisms by which HBx induces the MDR phenotype. This result was consistent with the data reported by Chen *et al.*<sup>[26]</sup>, who pointed out in his recent paper that inhibitors of the ERK/MAPK signal pathway could remarkably inhibited the expression of MDR-1. Barancik *et al.*<sup>[27]</sup> also suggested that SB203580, a specific inhibitor of the *p38*/MAPK pathway, could reverse the resistance of L1210/VCR cells mainly via P-glycoprotein dependent mechanisms. However, we could not exclude the possibility that ERK/MAPK activated by HBx could enhance the activity of P-glycoprotein (product of the *MDR-1* gene) by phosphorylation. P-glycoprotein is a phosphorylation substrate for a number of protein kinases including protein kinase C (PKC), MAPK, phospholipase C (PLC) *etc.*<sup>[28,29]</sup>. Therefore, phosphorylation of such MDR related proteins might be another function of ERK/MAPK pathway during MDR induced by

HBx protein. The influence of phosphorylation by such protein kinases as PKC or MAPK on the activity of MDR-1 protein and other MDR associated proteins is the target of our ensuing experiments in order to obtain a thorough understanding of the molecular mechanisms of interactions between HBx and MDR associated proteins.

In summary, MAPK pathway is involved in generation of MDR phenotype in hepatoma cells. The findings reported in this work might contribute to the understanding of pathogenic mechanisms involved in malignant phenotypes of HBV-associated hepatocellular carcinoma.

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