ORIGINAL ARTICLE

Effect of the HBV whole-X gene on the expression of hepatocellular carcinoma associated proteins

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Yu Zhang [a], Hongli Liu [b,c], Meiling Cui [d], Jinfeng Liu [a],
Ruitian Yi [a], Yinghua Niu [a], Tianyan Chen [a], Yingren Zhao [a,*]

[a] Department of Infectious Diseases, First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China
[b] Shaanxi Provincial Infectious Diseases Hospital, Xi'an 710061, Shaanxi Province, China
[c] Xi'an Eighth Hospital Affiliated to Xi'an Jiaotong University Health Science Center, Xi'an 710061, Shaanxi Province, China
[d] Zhengzhou Sixth People's Hospital, Zhengzhou 450061, He'nan Province, China

Background: The hepatitis B virus (HBV) pre-X gene resides upstream of the HBV X gene, and together they form the HBV whole-X gene. Although it has been evident that the HBV whole-X protein is involved in the development of hepatocellular carcinoma, its biological role and molecular mechanism remain largely unknown.
Methods: In this study, we subcloned the HBV whole-X gene and constructed a HBV whole-X expressing vector. After transfection of the HBV whole-X gene into HL-7702 cells, the profile of the differential cellular protein composition in the cells was analyzed by using two-dimensional electrophoresis coupled to matrix-assisted laser desorption/ionization-time of flight mass spectrometry.
Results: The results showed that 18 major proteins were differentially expressed in the cells transfected with or without the HBV whole-X gene. The expression of these genes was further confirmed by reverse transcription-polymerase chain reaction and Western blot analysis.
Conclusion: Our findings provide a new insight into the investigation of the pathological role that the HBV whole-X gene plays in the development of hepatocellular carcinoma and may lead to the design of novel strategies for the treatment of this disease.
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* Corresponding author. Department of Infectious Diseases, First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China.
E-mail address: zhaoyingren@mail.xjtu.edu.cn (Y. Zhao).

Introduction

Hepatitis B virus (HBV) infection may cause a chronic liver inflammation leading to an increased risk of liver cirrhosis and hepatocellular carcinoma (HCC).[1] The HBV DNA genome is partially double-stranded DNA, which is about 3200 bp long and contains four open reading frames (ORFs), the core (C), the surface (S), the polymerase (P), and the X gene.[2] The smallest X-ORF encodes the HBV X protein (HBx), which has transactivating functions and plays a crucial role in the development of HBV-associated HCC through interaction with host factors. Most studies have focused on the effective mechanism of the HBx protein in the pathology of HCC.[3,4] However, Loncarevic et al[5,6] cloned a replication competent of the HBV full-length genome in 1990 and found the presence of pre-X ORF before the X DNA region. Later, Takahashi et al[7,8] also reported the presence of pre-X ORF, which is 56 nucleotide triplets upstream of the promoter of X gene, and showed that the pre-X ORF was detected in 50% (19/38) of HBV DNA isolated from 40 HCC patients. Dong and colleagues[9,10] revealed that the pre-X gene and the X gene could be translated together and further characterized its transcriptional activity.

The whole-X gene, including the 168-bp pre-X and the 465-bp X genes, encodes the whole-X protein composed by 210 amino acids. Studies show that the pre-X gene is popular and well conserved in HBV genomes only when the start codon ATG is replaced by ACG (1205C → T), a missense mutation, resulting in the disruption of the pre-X gene translation. The information of the pre-X gene sequences is mainly generated from the samples of patients with HCC, chronic hepatitis, or acute fulminant hepatitis.[11] It has been reported that the whole-X protein binds with hepatocellular proteins in the yeast two-hybrid assay, indicating that the whole-X protein may play a role in regulating the signal transduction pathways in the hepatocytes through protein—protein interaction, which contributes to the pathogenesis of HCC.[12] However, the physiological role of the HBV whole-X gene remains largely unknown. In this study, we cloned the HBV whole-X gene and analyzed the altered protein expression profile in the cells with or without the whole-X gene by two-dimensional electrophoresis (2-DE) combined to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF)/TOF mass spectrometry (MS) analyses. To our knowledge, this is the first report about the global protein changes caused by the whole-X gene until now. Thus, the information gained herein may provide new information on how the whole-X gene participates in the pathogenesis of HBV-related HCC.

Methods

Cloning of HBV whole-X DNA and construction of plasmids

HBV DNA was extracted from the serum of patients with chronic hepatitis B using the phenol—chloroform extracting method. The HBV whole-X and X genes were amplified by polymerase chain reaction (PCR) using the corresponding primers as shown in Table 1, and then cloned into the pGEM-T vector. The full-length amplified whole-X gene and X gene were confirmed by DNA sequencing and subcloned into the pCMV-Tag2A vectors using *Eco*RI and *Xho*I digestion to construct pCMV-Tag2A-whole X and pCMV-Tag2A-X expression plasmids. The recombinants were verified by restriction enzyme digestion and DNA sequencing assays.

Cell culture and transfection

HL-7702 cells were cultured in Dulbecco's modified Eagle's medium high glucose (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA). When the cells were grown to 90% confluence, transfected with the recombinant plasmids pCMV-Tag2A-whole X and pCMV-Tag2A-X, respectively, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. Empty vector pCMV-Tag2A and pEGFP plasmids were also transfected into the cells as controls.

2-DE

The cells were harvested 48 hours after transfection, and the total cellular proteins were extracted by using the Ready-Prep protein extraction kit (Bio-Rad, Hercules, CA, USA). Briefly, the harvested cells were incubated on ice and sonicated for six cycles (each cycle included 6 seconds of sonication and a 10-second break), then centrifuged at 17,000 rpm (26,494×g) for 40 minutes; next, the supernatant was collected and cleaned up using the 2-D Clean Up kit (Bio-Rad) to remove the salt and impurities. The concentration of the total protein was quantified using the Bradford assay kit (Bio-Rad). The samples (0.4 mg protein per gel) were applied to 17 cm pH 3—10 nonlinear immobilized pH gradient (IPG) strips (Bio-Rad) by in-gel rehydration for 12 hours; then the rehydrated strips were focused as follows: 250 V for 1 hour; 1000 V for 1 hour; 10,000 V for 5 hours, and 10,000 V until 60,000 V/hour. After equilibration, 2-DE was carried out in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (18 cm × 20 cm × 1 mm) with 30 mA constant current. A modified silver staining method was used to stain the gels,[13] then the gels were scanned with a Bio-Rad GS-800 scanner. Next, the images were analyzed with the PDQuest software Version 6.1 (Bio-Rad) to compare the data between the gels of the transfected cells with or without the HBV whole-X gene. Each sample was processed three times to minimize experimental variations. Protein spots that showed reproducibly detected and significant differences of more than 2-folds ($p < 0.05$) in all three separate gels were picked out for the next MS analysis.

In-gel digestion

Silver-stained protein spots with significant intensity difference were cut from the 2-D gels, destained in 30 mM potassium ferricyanide and 100 mM sodium thiosulfate (1:1 v/v) for 20 minutes, and then washed with Milli-Q water. The destained spots were lyophilized after incubating in 0.2 M NH_4HCO_3 for 20 minutes. Each lyophilized protein spot was digested with 12.5 ng/mL trypsin in 25 mM

Table 1 The information of primers and PCR condition used in this study

Oligo name	Sequence (5′ to 3′)	Annealing temperature (°C)	Expected length (bp)
HBV whole-X gene	F[a]: CCAAGTGTTTGCTGACGCAACC	58[a]	630
	F[b]: GGAATTCCATGGGGCTTGGCTATTGG	56[b]	
	R: CCCTCGAGGGGTTAGGCAGAGGTGAAAAAG		
HBV X gene	F: GGAATTCCATGGCTGCTAGGCTGTGCTG	65	465
	R: CCCTCGAGGGGTTAGGCAGAGGGGAAAAAG		
RKIP	F: GAATAGACCCACCAGCAT	53	236
	R: CGTAAACCAGCCAGACAT		
KHSRP	F: GCACGGTTAGGAAAGCACAT	56	153
	R: TCCCTGTAGTTTTGAAGCGG		
PSMB4	F: CGTATATGCGAGTCAACGACA	54	330
	R: AAGCAGAGGCTGAGCCAAGA		
PDCD5	F: AGTAAGCCAACAAACAGAAAAGA	54	129
	R: CCTAGACTTGTTCCGTTAAGTTC		
β-Actin	F: AGGCCAACCGCGAAGATGACC	55	350
	R: GAAGTCCAGGGCGACGTAGCAC		

[a] To amplify the HBV whole-X gene from HBV DNA extracted from serum of patients with chronic hepatitis B.
[b] To amplify the HBV whole-X gene from pGEM-T—whole X vector.
HBV = hepatitis B virus; PCR = polymerase chain reaction.

NH_4HCO_3 overnight. Then, we extracted the peptides from the digested samples and dried the extracts by the vacuum centrifuge method.

MALDI-TOF/TOF MS analysis

All samples obtained from lyophilized trypsin peptides dried by a vacuum centrifuge and resuspended in 2 μL of 20% acetonitrile. The peptides were spotted on stainless-steel sample target plate and then subjected to 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA) equipped with a Nd:YAG laser (355 nm). Mass spectra for each peptide were obtained using an established setting as described previously.[14] The spectra were analyzed with the GPS-Explorer Software 3.6 (Applied Biosystems) and submitted to search the NCBI database to match the MS and MS/MS queries using the MASCOT search engine 2.2 (Matrix Science, Boston, MA, USA).

RNA extraction and reverse transcription-PCR

Total cellular RNAs were extracted from the cells after being transfected for 48 hours with recombinant plasmids by TRIzol reagent (Invitrogen). The expression of *RKIP*, *KHSRP*, *PSMB4*, and *PDCD5* genes was determined with reverse transcription (RT)-PCR using PrimeScript RT-PCR Kit (Takara Bio Inc, Tokyo, Japan) according to the manufacturer's instructions. The primer pairs are shown in Table 1.

Western blot analysis

The cells were collected 48 hours after transfection. Cell proteins were extracted using the whole-cell extraction buffer [50 mM Tris—HCl (pH 8), 2 mM ethylene glycol tetraacetic acid, 0.2 mM EDTA, 280 mM NaCl, 10% glycerol, and 0.5% NP-40] and quantified using the DC protein assay kit (Bio-Rad). Equal amounts of extracted proteins were run in

12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and then transferred to PVDF membranes (Millipore, Boston, MA, USA). Next, the membranes were blocked with 5% bovine serum albumin/ tris-buffered saline (BSA/TBS) for 1 hour and incubated overnight with a primary antibody at 4°C. For detection of *RKIP* (Raf-1 kinase inhibitory protein), *KHSRP* (KH-splicing regulatory protein), *PSMB4* (proteasome subunit beta type 4), and *PDCD5* (programmed cell death 5) expression, polyclonal rabbit anti-*RKIP* antibody (1:1000; Abcam, Cambridge, MA, USA), monoclonal mouse anti-*KHSRP* antibody (1:500; Abcam), monoclonal mouse anti-Proteasome subunit beta type 4 antibody (1:500; Abcam), and polyclonal rabbit anti-*PDCD5* antibody (1:1000; Abcam) were used. Antimouse or anti-rabbit Immunoglobulin G/Horseradish Peroxidase (Abcam) was used as secondary antibodies at 1:2000 dilutions.

Results

Construction of the HBV whole-X gene and the HBV X gene plasmids

The HBV whole-X genes and the HBV X genes were cloned, respectively, from the HBV DNA of chronic hepatitis B patients using a PCR cloning method. The cloned two DNA fragments (630 bp and 465 bp in size) were sequenced, and the sequences were confirmed to be identical to the HBV whole-X and X genes in the GenBank through a nucleotide blast analysis. The HBV whole-X and X genes were subcloned into pCMV-Tag2A using *Eco*RI and *Xho*I as shown in Fig. 1.

Expression of HBV whole-X gene in transfected cells

After transfection, the expression of the HBV whole-X and HBX genes was analyzed by RT-PCR and Western blot. Obviously, both genes were highly expressed in the transfected cells (Fig. 2A and B). To determine the transfection

efficiency, a plasmid with pEGFP was cotransfected with the HBV whole-X and HBX gene constructs into the cells. The result indicated that the transfection efficiency was >90% (Fig. 2C).

Protein profile in the cells with or without the HBV whole-X gene

The HBV whole-X gene and its function in the pathogenesis of HCC are largely unknown. We performed proteomic analysis to examine the expression of differential proteins in the transfected cells with or without the HBV whole-X gene. The cell extracts were fractionated by 2-DE, and the gels were subjected to silver staining (Fig. 3). After a comparison analysis using the PDQuest software (Bio-Rad), 29 protein spots with the alternation of density of more than two-fold ($p < 0.05$) from the HBV whole-X protein expressed cells were selected as significantly different candidate proteins for subsequent MALDI-TOF/TOF MS. Among the 29 samples, the identity of 18 proteins was successfully determined using this method. As listed in Table 2, the expression of 10 proteins was upregulated, whereas the expression of the other eight proteins was downregulated in the HBV whole-X gene expressed cells. Interestingly, these proteins are involved in diversiform processes of cell biology including apoptosis, signal transduction, and cell proliferation. For example, FK506-binding protein 3(FKBP3) is a novel regulator associated with the p53 signal pathway, and can induce the degradation of mouse double minute 2 (MDM2), leading to the activation of p53.[15] PDCD5 is an apoptosis-related gene with potent antitumor effects.[16] The RKIP may regulate many kinds of important signaling pathways. Specifically, RKIP inhibits the Ras-Raf-1-MEK1/2-ERK1/2 pathway through binding to Raf-

1 kinase. KHSRP has four KH-DNA binding domains responsible for binding to the FUSE sequence with great affinity and participates in the transcriptional activity of c-myc. Our data suggest that the HBV whole-X may promote the development of liver cancer by downregulating the expression of RKIP, an inhibitor of the Ras-Raf-1-MEK1/2-ERK1/2 pathway, and upregulating the expression of KHSRP, which is associated with the activity of c-myc, a well-known oncogene, in addition to suppressing apoptosis by inhibiting the expression of FKBP3 and PDCD5.

We also noted that PSMB4 was found significantly upregulated in whole-X and X gene transfected cells as shown in Fig. 4. PSMB4 has various proteolytic activities, and involved in the process of DNA repair and splicing, angiogenesis, apoptosis, and the removal of abnormal or damaged proteins in cells.

Validation of identified proteins

To validate the results from 2-DE and MS, the differential expression of the proteins in the cells with or without the HBV whole-X and HBV X genes was determined using RT-PCR and Western blot. As shown in Fig. 5A and B, the expressing level of RKIP and PDCD5 was downregulated, whereas KHSRP and PSMB4 expressions were upregulated in cells transfected with the HBV whole-X, which is consistent with the results of the proteomic analysis.

Intriguingly, although the RKIP expression was reduced in the cells transfected with either the HBV whole-X or the HBV X gene compared to that in the control cells, its level in the cells with the HBV X gene was significantly decreased compared to the HBV whole-X cells. However, the expression of KHSRP and PSMB4 was dramatically increased in the cells transfected with either of the whole-X cells and HBV X genes. The physiological significance of the altered expression of these proteins in the cells with either the HBV whole-X or the HBV X gene remains to be further investigated.

Discussion

It has been well documented that chronic HBV infection may lead to severe liver disease including fulminant hepatitis, liver cirrhosis, and HCC. HBV DNA integrates into the chromosomal genome of human hepatocytes, which is recognized to play a crucial role in the pathogenesis of the HCC.[17] The mechanisms by which HBV leads to hepatocyte malignant transformation is not clear. Although a line of existing evidence suggests a pathogenetic role for the HBx protein produced from the HBV X gene, studies have revealed the existence of a HBV pre-X gene that may contribute to the development of HBV-related acute fulminant hepatitis and HCC. The HBV pre-X and HBV X genes are combined to form the whole-X gene whose roles are not clear in HBV infectious diseases. In this study, we systematically analyzed the profiles of the differential cellular protein expression in the cells with or without the whole-X genes, and identified several targeting molecules of the gene in the cells by using 2-D gels, followed by MS analysis. The present study is the first to screen
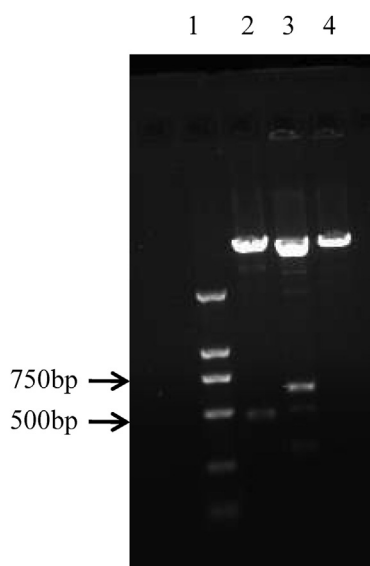

Lane 1: Marker 2000; Lane 2: pCMV-Tag2A-HBX;
Lane 3: pCMV-Tag2A-whole X; Lane 4: pCMV-Tag2A

Figure 1. Verification of the recombinant plasmids by restriction enzyme digestion.
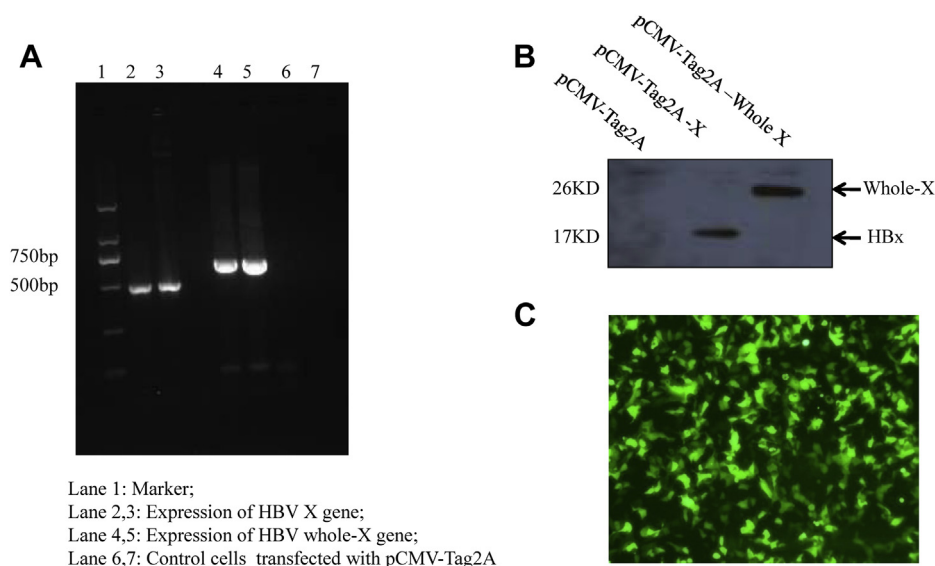
Figure 2. Expression of hepatitis B virus (HBV) whole-X and X gene in transfected cells. HL-7702 cells were transfected with pCMV-Tag2A, pCMV-Tag2A-X, and pCMV-Tag2A-whole X plasmids. (A) The transcriptional level of the genes expression were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). (B) The protein level of expression was analyzed by Western blot. (C) pEGFP was used to check transfection efficiency (×200).
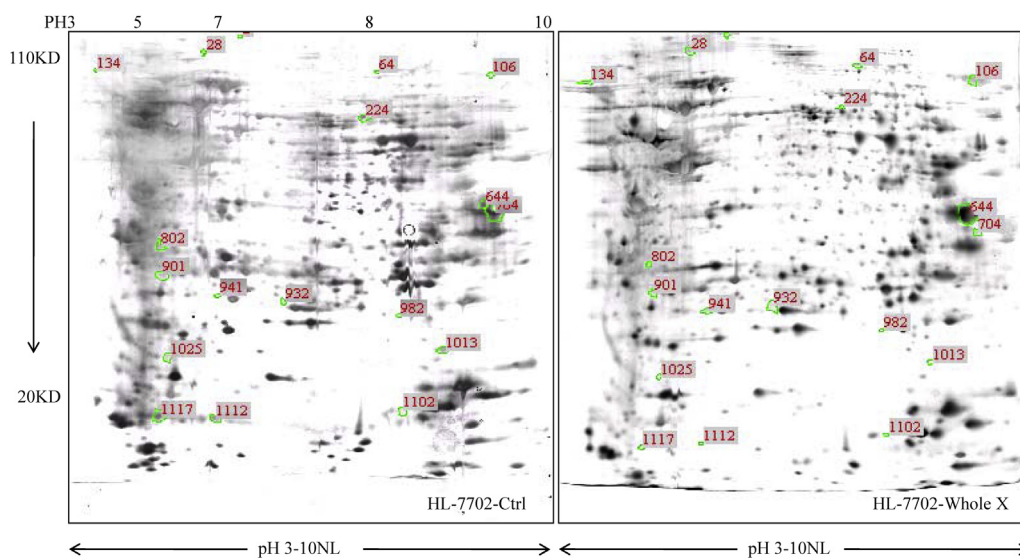
Figure 3. Representative 2-DE gel images in transfected cells with recombinants. Total protein extracts from the cells with pCMV-Tag2A (left) and whole-X (right) recombinants, were separated in the first dimension in IPG strips, then in the second dimension by 12% SDS-PAGE and stained using the silver stain method. Differentially density of protein spots that were identified by MS is indicated with circles. Information on 18 spots identified successfully by mass spectrometry is listed in Table 2. 2-DE = two-dimensional electrophoresis; IPG = immobilized pH gradient; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

differentially expressed proteins caused by HBV whole-X gene using comparative proteomic approaches.

The differential expression of a total of 18 proteins was found in the cells after overexpression of the whole-X gene. It is not surprising that these proteins are involved in apoptosis, signaling transduction, and protein turnover. The expression of FKBP3 and *PDCD5*, both of which are involved in the p53 pathway, was downregulated in the cells transfected with the whole-X gene, suggesting that suppression of apoptosis in liver cells may contribute to the pathogenesis of liver cancer. Indeed, studies have revealed that FKBP3 is a nuclear DNA-binding protein with a peptidylprolyl *cis*–*trans* isomerase activity,[18] and knock-down of endogenous FKBP3 increases the level of MDM2, leading to reductions of p53 and its downstream effectors p21.[15] It has been believed that *PDCD5* is a positive regulator in the p53 pathway by interacting with p53.[19] A decrease in *PDCD5* production is overtly able to reduce the activity of p53, resulting in the suppression of apoptosis, which promotes tumor progression. It has been reported that *PDCD5* also

**Table 2** List of identified increased or decreased proteins in the HBV whole-X gene expressed cells

| Accession no.	Protein description	Gene name	Function	Theoretical Mr/pI	No. of matched peptides	Protein Score	Coverage (%)	Alteration
gi|4503727	Peptidyl-prolyl cis—trans isomerase FKBP3	FKBP3	Protein folding	25,218.3/9.29	10	184	36	3.06↓
gi|4505621	Raf kinase inhibitory protein	RKIP	Signal transduction	21,157.7/7.01	11	327	63	2.98↓
gi|4759224	Programmed cell death protein 5	PDCD5	Apoptosis	14,276.3/5.77	7	137	57	2.85↓
gi|157412270	Heterogeneous nuclear ribonucleoprotein M	HNRNPM	mRNA splicing	73,857.5/8.94	35	668	46	2.56↓
gi|7542837	Medium-chain acyl-CoA dehydrogenase	ACADM	Lipid metabolism	47,014.8/8.61	16	362	32	2.41↓
gi|1100209	Transcription factor ZFM1	SF1	Transcription regulation	62,220.1/9.4	8	93	39	2.36↓
gi|13937981	Peptidylprolyl isomerase A	PPIA	Regulation of viral genome replication	18,228/7.68	7	201	54	2.08↓
gi|5031777	Isocitrate dehydrogenase (NAD) subunit alpha	IDH3A	Binding	40,022.2/6.47	9	183	26	2.06↓
gi|54648253	KHSRP protein	KHSRP	Transcription regulation	73,307.5/8	27	753	45	2.93↑
gi|48145757	Proteasome subunit beta type-4	PSMB4	Apoptosis	29,228.5/5.7	10	368	46	2.83↑
gi|4507645	Triosephosphate isomerase isoform 1	TPI1	Metabolism	26,937.8/6.45	18	489	67	2.79↑
gi|55664661	Voltage-dependent anion channel 2	VDAC2	Binding	30,842.1/8	11	312	50	2.68↑
gi|23308577	D-3-Phosphoglycerate dehydrogenase	PHGDH	Metabolism	57,355.7/6.29	19	529	41	2.63↑
gi|45767717	Histone cluster 1, H2bm	HIST1H2BM	Binding	14,079.7/10.39	8	143	58	2.57↑
gi|4502549	Calmodulin	Calm1	Receptor binding	16,826.8/4.09	2	125	25	2.44↑
gi|23308751	3-Hydroxyisobutyrate dehydrogenase	HIBADH	Metabolism	35,704.9/8.38	4	94	20	2.39↑
gi|2204207	Glutathione S-transferase	GST	Metabolism	23,595.1/5.43	7	361	48	2.28↑
gi|31645	Glyceraldehyde-3-phosphate dehydrogenase	GAPC	Binding	36,202.4/8.26	16	422	52	2.12↑

HBV = hepatitis B virus.

induces cell apoptosis via inhibiting the Ras/Raf/MEK/ERK signaling pathway.[20] Downregulation of *PDCD5* expression has been found in several types of human tumors, and changing of the protein level is used for the prognosis of cancer progression.[21—24] It has been well established that *RKIP* inhibits the MAPK pathway by disrupting the interaction between the Raf-1 and MEK the upstream components of ERK.[25,26] The activation of the Ras/Raf/MEK/ERK signaling pathway is associated with many kinds of human cancers, for example, breast cancer and renal cell carcinoma.[27,28] Studies have shown that *RKIP* mRNA and protein were downregulated in HCC.[29,30] As a result, this leads to the constitutive activation of the ERK/MAPK pathway in HCC tumors. Furthermore, *RKIP* was also found to be downregulated during HBx-mediated

hepatocarcinogenesis.[31,32] It is possible that the function of the HBV whole-X in hepatocytes is to suppress apoptosis and promote cell proliferation, which leads to the development of HCC; however, further investigation is necessary to evaluate the results.

PSMB4 is involved in the proteasome pathway. Studies have linked the proteasomal proteins to tumorigenesis. The previous studies proved that the ubiquitin—proteasome pathway plays a crucial role in intracellular protein degradation, such as cell cycle regulatory proteins. An increased level of proteasomal proteins was found in leukemia cells[33] and samples from patients with renal carcinoma[34] and breast cancer.[35] Evidence has shown that the proteasome is a possible cellular target of HBx.[36] HBx interacts with the subunits of proteasome,[37—39] impacting hepadnavirus
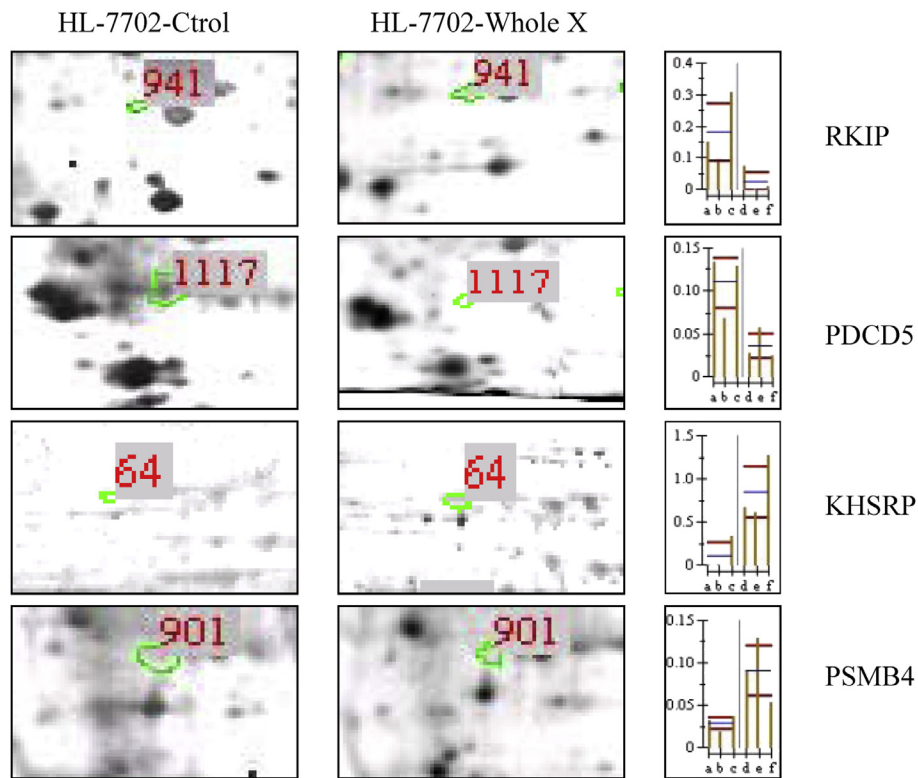
Figure 4. Analysis of the proteins of interest. Protein extracts from the whole-X cells and control were compared in two-dimensional electrophoresis (2-DE) gel. The proteins of interest are selected and circled. The histograms of expression kinetics are shown on the right.
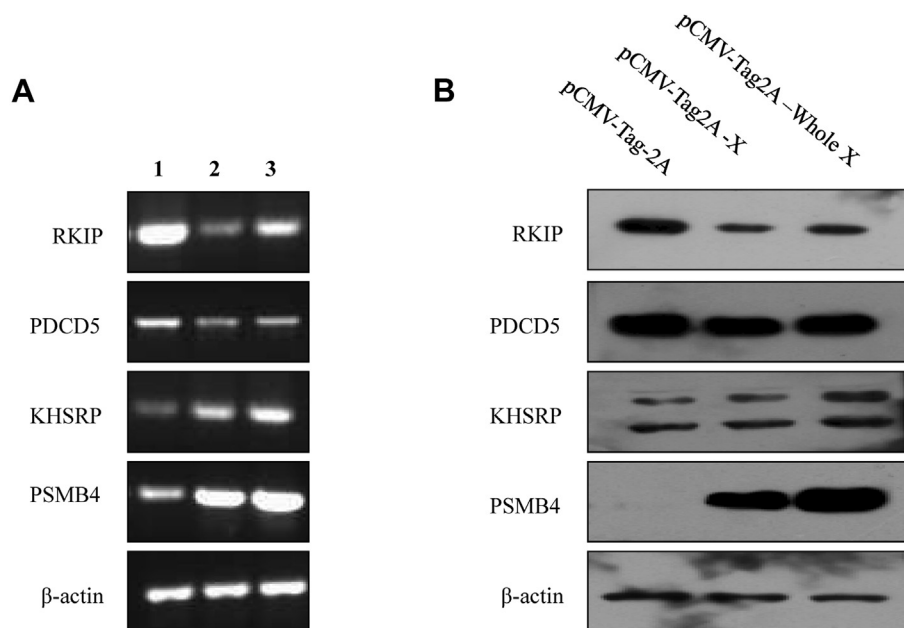
Figure 5. Verification of 2-DE results by RT-PCR and Western blot. (A) In RT-PCR analysis, the mRNA expressions of four proteins were analyzed in vector control cells (1), HBx cells (2), and whole X cells (3). (B) Western blot assay identified differential proteins in transfected HL-7702 cells with different plasmids. β-Actin is included as an internal control. 2-DE = two-dimensional electrophoresis; HBx = HBV X protein; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

replication.[40] Our data revealed that *PSMB4* in the cells overexpressing the HBV whole-X and HBx gene is significantly upregulated, suggesting that HBV infection promoting the pathogenesis of HCC may be through accelerating protein turnover as well.

Taken together, our results provide new insight into the investigation of molecular mechanism by which the HBV whole-X gene contributes to the development of HCC. The information may be useful for the design of therapeutic strategies for HCC and other liver diseases caused by HBV infection.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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