Interferon-α inhibits cell migration and invasion and induces the expression of antiviral proteins in Huh-7 cells transfected with hepatitis B virus X gene-expressing lentivirus

QIAN YANG, XIAO-PENG LI, YUAN-BIN ZHONG, TIAN-XIN XIANG and LUN-LI ZHANG

Department of Infectious Disease, The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, P.R. China

Abstract. Hepatitis B virus (HBV) X protein (HBx) serves an important role in HBV infection and the development of HBV-related liver cancer. Interferon-α (IFN-α) is used to treat patients with HBV; however, the role of IFN-α in the development of HBV-related liver cancer remains unclear. The present study established a new HBV-related liver cancer model (Huh-7-HBx) by transfecting the hepatoma cell line Huh-7, with HBx-expressing lentivirus. Following IFN-α treatment, cell viability, migration and invasion, as well as the expression of antiviral proteins in Huh-7-HBx, were subsequently determined. The results demonstrated that HBx-expressing lentivirus had no significant effect on cell viability but promoted the migration and invasion of Huh-7 cells. The expression of the antiviral genes IFN α and β receptor subunit 1 (IFNAR1), IFNAR2, IFN-stimulated gene factor 3, double-stranded RNA-activated protein kinase and ribonuclease L, was also increased. Following treatment of Huh-7-HBx cells with IFN-α, the expression of antiviral genes was increased at the level of transcription and translation, whereas cell migration and invasion was decreased. The present study suggests that IFN-α may attenuate the development of HBV-related liver cancer by reducing cell migration and invasion and promoting the expression of antiviral proteins.

Introduction

Hepatitis B virus (HBV) is an infectious disease that poses a serious threat to human health. It is demonstrated that sexual promiscuity, transfusion of unscreened blood, reusing or sharing of syringes between injection in drug users are the predominant associated risk factors (1,2). The World Health Organization estimates that there are ~350 million people worldwide infected with HBV, which may develop into chronic hepatitis B, liver fibrosis, liver sclerosis or liver cancer (3,4). The estimated worldwide mortality is 0.5 to 1.2 million fatalities a year (1). However, t here are currently no effective treatments for HBV-related liver cancer.

Interferon-α (IFN-α) is an antiviral cytokine that has a broad spectrum of action, exhibits high activity and indirect and species specificity (5,6). IFN-α exerts its antiviral activity via activation of the Janus kinase/signal transducer and activator of transcription (JAK-STAT) signaling pathway (7,8). In addition, IFN-α inhibits tumor development by decreasing cell viability, promoting cell apoptosis and attenuating tumor angiogenesis (9-11). IFN-α serves a role in immune surveillance and regulation by enhancing the immune function of T- and B-lymphocytes, natural killer cells and macrophages to enhance the body's ability to kill cancer cells and tumor cells infected by the virus (12,13). The effect of IFN-α on anti-viral, anti-tumor and immune regulation indicates that IFN-α may be used as to treat patients with HBV-related liver cancer. However, the role served by IFN-α regulation in the development of HBV-related liver cancer remains unknown.

Hepatitis B X protein (HBx), encoded by HBV DNA, serves an important role during the development of chronic hepatitis B, liver cirrhosis and liver cancer (14). Therefore, the current study established a novel HBV-related liver cancer model by transfecting the hepatoma cell line Huh-7 with HBx-expressing lentivirus, which has been previously studied (15-18) and subsequently investigated the effect of IFN-α on the growth of cancer cells to identify its potential as a drug for treating HBV-related liver cancer.

Materials and methods

Cell culture. The human hepatoma cell line Huh-7 (The Cell Bank of Type Culture Collection of Chinese Academy of Sciences; Wuhan, China) was cultured in Dulbecco's Modified Eagle medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 80 U/ml penicillin and 80 µg/ml streptomycin (HyClone; GE Healthcare Life Sciences). The cells were incubated in 5% CO_2 at 37°C. 1,000 IU/ml of IFN-α (Sigma-Aldrich; Merck KGaA; Darmstadt, Germany) was used to treat the cells in the following experiments.

Correspondence to: Professor Lun-Li Zhang, Department of Infectious Disease, The First Affiliated Hospital of Nanchang University, 17 Yongwaizheng Street, Nanchang, Jiangxi 330006, P.R. China
E-mail: z_lunli@126.com

Transfection of HBx-expressing lentivirus into Huh-7 cells. HBx-expressing lentivirus was produced from pLenti6.2/V5-DEST plasmid (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with the second generational system. Briefly, the packaging plasmids were transformed into 293T cells with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Lentivirus was harvested 72 h after transfection, and then the titer was determined as described previously (19). Huh-7 cells were transfected with $3x10^7$ infectious units per milliliter of HBx-expressing lentivirus (Novobio Scientific, Inc., Shanghai, China) on a 96-well plate. The control cells were transfected with the same concentration of empty lentivirus. Medium was replaced 24 h following transfection, and subsequent experiments began 24 h post transfection.

Treatment groups. The following four groups were used in the present study: Control (no treatment); IFN-α only, Huh-7 cells were treated with 1,000 IU/ml of IFN-α for 24 h at 37°C; HBx infected cells, Huh-7 cells were transfected with $3x10^7$ infectious units per milliliter of HBx-expressing lentivirus for 24 h at 37°C; HBx+IFN-α group, after the transfection of HBx-expressing lentivirus for 24 h, Huh-7 cells were treated with 1,000 IU/ml of IFN-α for an additional 24 h.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells were washed with cold PBS three times and harvested following centrifugation at 3,000 x g at 4°C for 5 min. Total RNA was extracted using RNAiso Plus (Takara Bio., Dalian, China) according to the manufacturer's instructions and subjected to electrophoresis on 1.5% native agarose gel for integrity and quality analysis. A total of 1 µg RNA was used for cDNA synthesis with a PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan) following the manufacturer's protocol. A total of 1 µl 20-fold-diluted cDNA was used as a template for qPCR, which was performed using the Bio-Rad Detection system and SYBR Green qPCR SuperMix (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol with the corresponding primers (Table I). PCR conditions consisted of: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Relative expression was determined following normalization to the reference gene GAPDH. RT-qPCR data were collected from three independent biological replicates and analyzed with the $2^{-\Delta\Delta CT}$ method (20).

Western blot analysis. Antibodies against β-actin (catalogue no., ab8227), IFN α and β receptor subunit 1 (IFNAR1) (catalogue no., ab45172), IFNAR2, interferon-stimulated gene factor 3 (ISGF3) (catalogue no., ab56070), double-stranded RNA-activated protein kinase R (PKR) (catalogue no., ab32506) and ribonuclease L (RNase L) (catalogue no., ab191392) were purchased from Abcam (Cambridge, UK). Cells were lysed with Cell lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) and centrifuged at 10,000 x g at 4°C for 15 min. Supernatant lysates were harvested and the concentration was determined by Bradford method with Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, China). Subsequently, 30 µg of proteins were loaded per lane and subjected to 10% SDS-PAGE. Proteins in the gel were transferred to a nitrocellulose membrane and the membrane was blocked with 5% milk powder at 4°C overnight. Primary antibodies were diluted 1:1,000 in TBST buffer and incubated for 2 h at room temperature. Following incubation with primary antibodies, the membrane was washed three times with TBST. Horseradish peroxidase conjugated secondary antibody (anti-rabbit IgG) (catalogue no., ab6721; Abcam) was diluted 1:5,000 in 5% milk powder and incubated for 2 h at room temperature. The blotting membrane was then washed three times with TBST. Target bands were detected using ECL regents (Beyotime Institute of Biotechnology; catalogue no., P0018) and quantitatively analyzed using Quantity one software version 4.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). β-actin was used as the reference gene.

Detection of cell viability using an MTT assay. Following transfection, a total of $1x10^5$ Huh-7 cells/well were seeded in a 96-well plate and allowed to adhere and spread for 24 h. Huh-7 cells in the IFN-α alone and HBVx-expressing lentivirus + treatment with IFN-α groups were treated with IFN-α. A total of 10 µl MTT solution (5 mg/ml in PBS) was subsequently added to the cell medium of cells in all groups and incubated for 4 h at 37°C. The culture supernatant was subsequently removed, 100 µl dimethylsulphoxide was added and the solution was shaken for 10 min. Absorbance was measured at a wavelength of 490 nm.

Cell scratch test. A total of $1.6x10^5$ Huh-7 cells were seeded in a 6-well plate and allowed to grow until 80% confluence was reached. A micropipette tip was used to gently scratch a line of cells off the plate. The cell plate was washed three times with PBS to remove any remaining scratched cells. Following treatment, cells were cultured for an additional 24 h in serum-free DMEM. Cell migration into the scratch site was measured using an inverted IX81 Olympus microscope (Olympus Corporation of the Americas; Center Valley, PA, USA) and ImageJ software version 1.41o (National Institute of Health; Bethesda, MD, USA).

Cell invasion test. A suspension of $0.5x10^6$ cells was placed in each well of the lower chamber of a 24-well plate. Cells were cultured in DMEM containing 10% fetal bovine serum. The upper chamber Matrigel culture insert was then placed on top of the lower chamber. A total of $1x10^5$ of cells were added on top of the Transwell membrane in the upper chamber, cultured in serum free DMEM. Invasion chambers were incubated for 48 h in 5% CO_2 at 37°C. Following incubation, noninvasive cells were removed by scraping the upper surface of the Matrigel membrane using a cotton swab. Invading cells on the lower surface of the membrane were fixed with 4% paraformaldehyde for 15 min at room temperature, washed with PBS and stained with Giemsa solution for 10 min at room temperature. The stained membrane was photographed using an inverted IX81 Olympus microscope (Olympus Corporation of the Americas) and the number of cells was counted.

Statistical analysis. Data are expressed as the mean ± standard deviation based on three independent biological replicates. Statistical analysis was performed using SPSS 19.0

Table I. Primers for reverse transcription-quantitative polymerase chain reaction.

Genes	Forward (5'-3')	Reverse (5'-3')
HBx	GTCAACGCATAGTGGGTCT	CCTTGTAGGTTGGCGAGA
IFNAR1	GTAAAGGTGGACATCAGTG	AGAATCTGGTAAGGGAAA
IFNAR2	AAATGCACCCTCCTTCCA	AGCCCTTAGCGAGACCTT
ISGF3	TGGCATTTCTGACTTTCTCC	GGGCTATGGTAATGTGGGTA
PKR	CGTGCCTGGATTGAGAAA	CATCACTGCCGAACATTA
RNaseL	GGAAGCGAGGAGCACAAG	TGGGCACATTCAGGAACT
GAPDH	AGGGCTGCTTTTAACTCTGG	CCCCACTTGATTTTGGAGGG

HBx, hepatitis B X protein; IFNAR1, interferon α and β receptor subunit 1; IFNAR2, interferon α and β receptor subunit 2; ISGF3, interferon-stimulated gene factor 3; PKR, double-stranded RNA-activated protein kinase R; RNaseL, ribonuclease L.
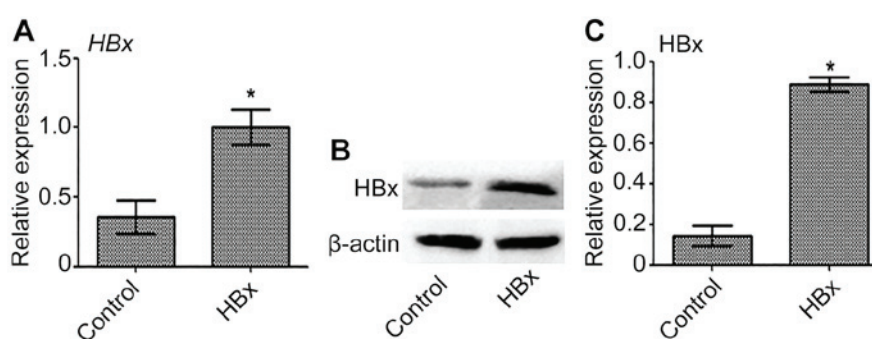

Figure 1. Expression of HBx mRNA and protein following the transfection of Huh-7 cells with HBx-expressing lentivirus. (A) Expression of HBx mRNA. (B) Western blot evaluating HBx expression. (C) Quantification of HBx protein expression. Data are expressed as the mean ± standard deviation based on three independent biological replicates. Statistically significant differences were determined using a Student's t-test. *P<0.05 vs. control. HBx, hepatitis B X protein.

(IBM Corp., Armonk, NY, USA). Student's t-test was used to conduct a pairwise comparison. Multiple comparisons were tested using one-way analysis of variance, followed by a Tukey honest significant difference test. P<0.05 was determined to indicate statistically significant difference.

Results

Transfection of Huh-7 cells with HBx-expressing lentivirus. Transfection of Huh-7 cells with HBx-expressing lentivirus was performed to establish a novel HBV-related liver cancer model (Huh-7-HBx). The expression of HBx mRNA and protein in Huh-7-HBx cells was significantly upregulated compared with the control (P<0.05; Fig. 1). Therefore, HBx was successfully overexpressed in Huh-7 cells.

Cell viability is not affected by the transfection of Huh-7 cells with HBx-expressing lentivirus or by treatment with IFN-α. Huh-7 cell viability was measured using an MTT assay following the transfection of Huh-7 cells with HBx-expressing lentivirus and IFN-α treatment. The effect of IFN-α treatment alone on Huh-7 cell viability compared with the control group was not significant (Fig. 2). Similarly, the difference in cell viability in the Huh-7-HBx and Huh-7-HBx+IFN-α treatment groups compared with the control was not significant. IFN-α treatment only slightly inhibited the viability of Huh-7-HBx

cells. These results suggest that IFN-α does not affect cell viability in HBV-related liver cancer.

IFN-α inhibits HBx-induced cell migration in Huh-7 cells. Migration is an important characteristic of cancer cells; therefore cell migration was examined in the Huh-7-HBx and Huh-7-HBx+IFN-α treatment groups to determine the curative function of IFN-α in HBV-related liver cancer (Fig. 3). Cell migration was decreased (P<0.05) in the IFN-α treatment alone group (Fig. 3B) and increased (P<0.05) in the Huh-7-HBx group (Fig. 3C) compared with the control (Fig. 3A). There was a decrease (P<0.05) in cell migration in the Huh-7-HBx+IFN-α treatment group (Fig. 3D) compared with the control, but not to the same extent as the decrease observed in the IFN-α treatment alone group. Quantitative analysis of cell migration in each of the groups supported these observations (Fig. 3E). This suggests that IFN-α may inhibit cell migration in HBV-related liver cancer.

IFN-α inhibits HBx-induced Huh-7 cell invasion. Cell invasion indicates tumor carcinogenesis, thus the HBx-regulated liver cancer model was used to determine the curative function of IFN-α in HBV-related liver cancer (Fig. 4). Huh-7 cell invasion was decreased (P<0.05) in the IFN-α treatment alone group (Fig. 4B) and increased (P<0.05) in
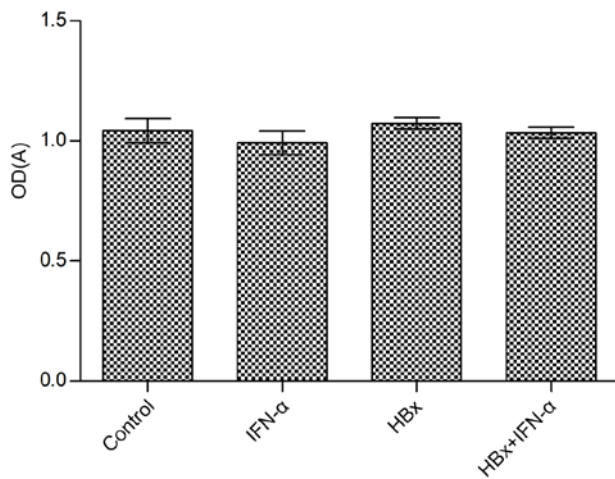
Figure 2. Cell viability following transfection of Huh-7 cells with HBx-expressing lentivirus and subsequent treatment with IFN-α. Data are expressed as the mean ± standard deviation. Statistically significant differences were determined using analysis of variance, followed by the Tukey honest significant difference test. HBx, hepatitis B X protein; IFN-α, interferon-α; OD, optical density; (A), absorbance.
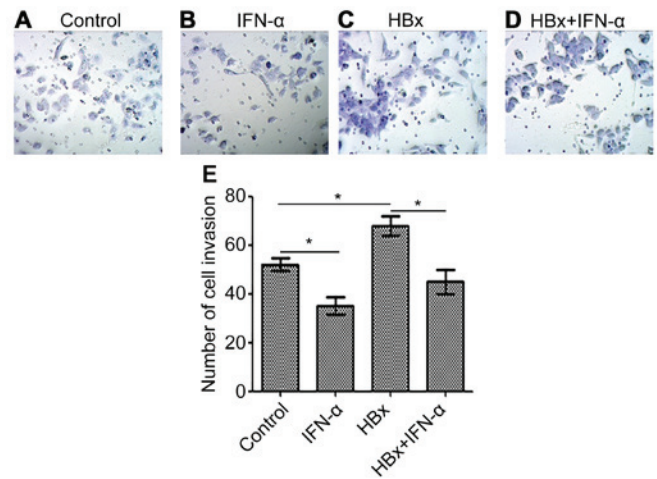

Figure 4. Cell invasion following the transfection of Huh-7 cells with HBx-expressing lentivirus and subsequent IFN-α treatment. Cell invasion in the (A) control, (B) IFN-α, (C) HBx and (D) HBx + IFN-α groups was indicated by Giemsa staining. (E) Quantification of cell invasion. Data are expressed as the mean ± standard deviation based on three independent biological replicates. Statistically significant differences were determined using analysis of variance, followed by the Tukey honest significant difference test. *P<0.05 as indicated. HBx, hepatitis B X protein; IFN-α, interferon-α.

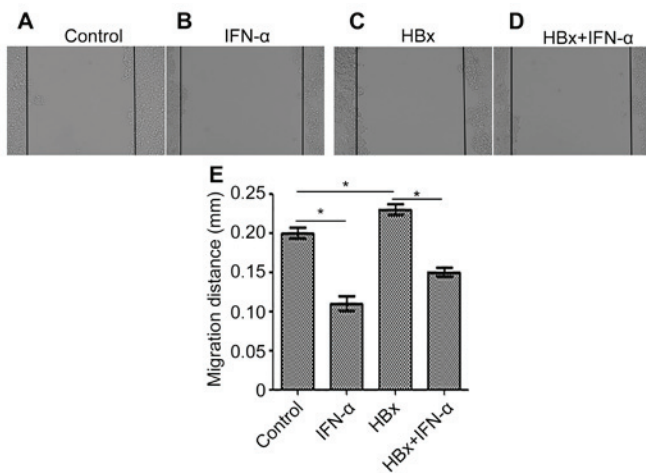Figure 3. Cell migration following the transfection of Huh-7 cells with HBx-expressing lentivirus and subsequent IFN-α treatment. Cell migration in the (A) control, (B) IFN-α, (C) HBx and (D) HBx + IFN-α groups was determined using a cell scratch test. The images were acquired using a phase contrast microscope at x10 magnification. Scale bar indicates 50 μm. (E) Quantification of cell migration. Data are expressed as the mean ± standard deviation based on three independent biological replicates. Statistically significant differences were determined using analysis of variance, followed by a Tukey honest significant difference test. *P<0.05 as indicated. HBx, hepatitis B X protein; IFN-α, interferon-α.

the Huh-7-HBx group (Fig. 4C) compared with the control (Fig. 4A). There was a decrease (P<0.05) in cell invasion in the Huh-7-HBx+IFN-α treatment group (Fig. 4D) compared with the control, but not to the same extent as the decrease observed in the IFN-α treatment alone group. Quantitative analysis of cell invasion was consistent with these results (Fig. 4E). This indicates that IFN-α may reduce cell invasion in HBV-related liver cancer.

IFN-α promotes the expression of antiviral genes during transcription and translation in Huh-7-HBx cells. IFNAR1,

IFNAR2, ISGF3, PKR and RNase L are important antiviral genes that exhibit anti-HBV effects (21-25). Therefore, the expression of the antiviral genes was examined in a HBx-regulated liver cancer model to determine the curative function of IFN-α in HBV-related liver cancer. RT-qPCR demonstrated that the expression of IFNAR1 (Fig. 5A), IFNAR2 (Fig. 5B), PKR (Fig. 5C), RNaseL (Fig. 5D) and ISGF3 (Fig. 5E) mRNA was significantly increased (P<0.05) in the IFN-α treatment only group compared to the control. mRNA levels of these antiviral genes were upregulated (P<0.05) in the Huh-7-HBx group compared with the control group however, not to the extent of the increase demonstrated in the IFN-α treatment only group. The most significant increase in expression of antiviral gene mRNA, compared with the control, was in the Huh-7-HBx+IFN-α treatment group. The protein expression of these antiviral genes was consistent with this (Fig. 5F-K). These results suggest that IFN-α increases the mRNA and protein levels of antiviral genes in HBV-related liver cancer.

Discussion

HBV infection is a major cause of primary liver cancer (12,26). The present study demonstrated that the transfection of Huh-7 cells with HBx, a protein encoded by HBV DNA, had no effect on cell viability but promoted cell migration and invasion, which is consistent with the results of a previous study (27).

IFN-α is currently used as a first-line antiviral drug to treat chronic hepatitis B (CHB) (28,29). It is an effective treatment of HBV due to its antiviral function and immunomodulatory effects (30-34). IFN-α does not directly kill or inhibit HBV; however, its antiviral effect is facilitated by its binding to the cell membrane receptor IFNAR1, which leads to the production of antiviral proteins that inhibit HBV replication (35,36). Therefore, IFNAR1 serves a role in the progression of CHB. The binding of IFN-α to its receptor IFNAR1 activates Janus

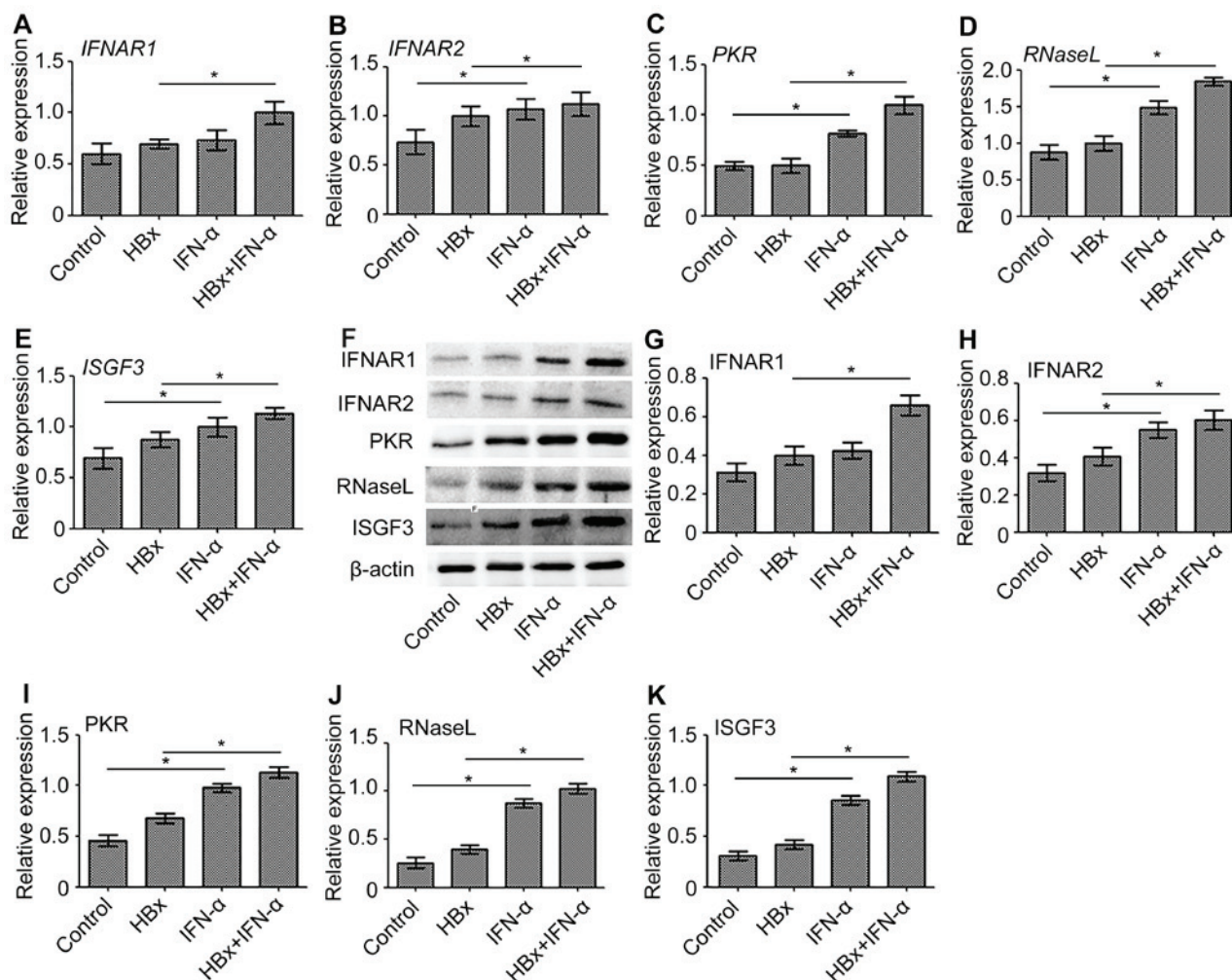EXPERIMENTAL AND THERAPEUTIC MEDICINE 14: 5924-5930, 2017

Figure 5. Expression of antiviral gene mRNA and protein following the transfection of Huh-7 cells with HBx-expressing lentivirus and subsequent IFN-α treatment. The expression of (A) IFNAR1, (B) IFNAR2, (C) PKR, (D) RNaseL, (E) ISGF3 mRNA was determined using reverse transcription-quantitative polymerase chain reaction. The protein expression of antiviral genes was detected using (F) western blot analysis and quantified for (G) IFNAR1, (H) IFNAR2, (I) PKR, (J) RNaseL and (K) ISGF3 using Quantity one software. Data are expressed as the mean ± standard deviation based on three independent biological replicates. Statistically significant differences were determined using analysis of variance, followed by the Tukey honest significant difference test. *P<0.05 as indicated. HBx, hepatitis B X protein; IFN-α, interferon-α; IFNAR1, interferon α and β receptor subunit 1; IFNAR2, interferon α and β receptor subunit 2; PKR, double-stranded RNA-activated protein kinase R; RNaseL, ribonuclease L; ISGF3, interferon-stimulated gene factor 3.

kinase-JAK1 and non-receptor tyrosine-protein kinase TYK2, which leads to the phosphorylation of signal transducer and activator of transcription (STAT) 1 and STAT2 (37,38). STAT1 and STAT2 then form a heterodimer and bind to interferon regulatory factor 9 (IRF-9) to form ISGF3. ISGF3 translocates from the cytoplasm to the nucleus and binds to the IFN stimulated regulatory element to promote the transcription of antiviral genes, such as PKR (39-41). Previous studies have demonstrated that following treatment with IFN-α, the expression of STAT2, IRF-9, and PKR is significantly increased in HepG2 and HepG2.2.15 cells (42,43). There was also a decrease in HBV DNA titer in HepG2.2.15 cells, which suggests that the JAK-STAT pathway serves a major role in IFN-α-inhibited HBV replication. Furthermore, the expression of ISGF3 protein and PKR mRNA was significantly decreased following inhibition of the IFN pathway and the titer of HBV DNA in the supernatant of HepG2.2.15 cells was not significantly decreased. These results indicate that ISGF3 is an important regulatory factor of the pathway (25,44).

IFN-α activates the JAK-STAT signaling pathway by binding to IFNARs on the cell surface, thereby facilitating the transcription and expression of antiviral genes, including PKR (45) and RNaseL (46). The present study demonstrated that IFN-α significantly increases the expression of the antiviral genes IFNAR1, IFNAR2, PKR, RNaseL and ISGF3 in Huh-7 cells transfected with HBx-expressing lentivirus, suggesting that IFN-α may be developed as a novel therapeutic strategy to treat patients with HBV-related liver cancer.

In conclusion, the current study suggests that IFN-α attenuates the development of HBV-related liver cancer by reducing cell migration and invasion, as well as upregulating the expression of antiviral proteins.

Acknowledgements

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