

HBx-associated long non-coding RNA activated by TGF- β promotes cell invasion and migration by inducing autophagy in primary liver cancer

YUHEN ZHANG^{1*}, JUN LI^{2*}, SHUAI WANG¹, FAJI YANG¹,
YUAN ZHOU¹, YANG LIU¹, WEI ZHU³ and XIAOLEI SHI¹

¹Department of General Surgery, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing, Jiangsu 210008; ²Department of General Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui 230022; ³Department of Anesthesiology, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing, Jiangsu 210008, P.R. China

Received September 27, 2018; Accepted March 7, 2019

DOI: 10.3892/ijo.2019.4908

Abstract. Hepatitis B virus (HBV) x protein (HBx) has been reported as the primary pathogenic factor involved in HBV-related liver cancer; however, the mechanisms underlying how HBx promotes tumor-associated invasion and metastasis remain unclear. Long noncoding RNA activated by transforming growth factor (TGF)- β (lncRNA-ATB) is a novel oncogenic lncRNA stimulated by TGF- β , which is closely associated with the invasion and metastasis of liver cancer. In the present study, whether lncRNA-ATB was involved in HBx-mediated hepatocarcinogenesis was investigated. The expression of lncRNA-ATB in 26 primary liver cancer tissues and lentivirus transfected HBx-HepG2 cell lines was detected, and it was revealed that more advanced tumor-node-metastasis staging and increased expression of lncRNA-ATB in liver cancer

tissues were significantly associated with HBV infection. It was further demonstrated that the expression levels of lncRNA-ATB and TGF- β were elevated in HepG2 cells following HBx-vector transfection, which was accompanied with increased autophagy. Conversely, knockdown of lncRNA-ATB or TGF- β could suppress this effect. Furthermore, such suppression on autophagy in HepG2 cells could be alleviated by the induction of starvation. In addition, the invasive and migration abilities of HBx-HepG2 cells were increased compared with HepG2 cells, while knockdown of lncRNA-ATB or TGF- β could reduce these abilities. In conclusion, the results of the present study revealed that HBx was closely associated with oncogenic lncRNA-ATB. HBx-induced autophagy could upregulate the expression of TGF- β and lncRNA-ATB. This may be considered to be a potential mechanism underlying HBV-induced hepatocarcinogenesis.

Correspondence to: Professor Xiaolei Shi, Department of General Surgery, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, 321 Zhongshan Road, Nanjing, Jiangsu 210008, P.R. China
E-mail: njxl2000@163.com

Professor Wei Zhu, Department of Anesthesiology, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, 321 Zhongshan Road, Nanjing, Jiangsu 210008, P.R. China
E-mail: chzhuwei118@qq.com

*Contributed equally

Abbreviations: HBx, hepatitis B Virus protein x; lncRNA-ATB, long non-coding RNA activated by TGF- β ; TGF- β , transforming growth factor- β ; LC3, microtubule-associated protein light chain 3; siRNA, small interfering RNA; EBSS, Earle's balanced salt solution; DMEM, Dulbecco's modified Eagle media

Key words: HBx, liver cancer, lncRNA-transforming growth factor- β , autophagy, invasion, migration

Introduction

At present, primary liver cancer is one of the most common malignancies in the Chinese population and is the second leading cause of cancer-associated mortality in males (1,2). Chronic hepatitis B virus (HBV) infection is one of the most critical risk factors for liver cancer (3). Effective treatment strategies have been reported in the past few decades; however, clinical studies have demonstrated that the morbidity and mortality of HBV-associated liver cancer remains high, on account of an increased incidence of cancer metastasis and invasion (4). Previously, HBV X protein (HBx), encoded by HBV, which can alter the cell cycle, proliferation and apoptosis-associated target gene expression of hepatocytes, was considered to be an essential protein in the development of HBV-related liver cancer (5,6). Although, the specific mechanisms as to how HBx mediates hepatocarcinogenesis remain unclear.

Long noncoding RNA (lncRNA) is a type of noncoding RNA >200 bp in length, without the ability to encode proteins (7). LncRNAs can affect cell behavior; the abnormal expression or function of lncRNAs are firmly associated with abnormalities in cell status, differentiation, developmental

diseases and cancer (8-10). An increasing number of studies have reported that lncRNAs are involved in the development of liver cancer, including Hox antigenic intergenic RNA, hepatocellular carcinoma upregulated lncRNA (HULC), hepatocellular carcinoma upregulated EZH2-associated lncRNA, Dreh and UCF1, by affecting cell proliferation, apoptosis and metastasis (11-14). Numerous lncRNAs have been reported to be associated with HBx. For example, elevated lncRNA-HULC expression was observed in HBx-overexpressed liver cancer cell lines with upregulated cell proliferation, which could be due to the enhanced inhibition of tumor suppressor gene p18 located near HULC (15). In addition, HBx-long interspersed nuclear elements (LINE1), produced by the transcription of the HBx gene promoter region, was revealed to be expressed in the tumor tissues of patients with HBV-related liver cancer and was associated with poor prognosis (16). Cell experiments demonstrated that HBx-LINE1 could promote tumor cell colony formation, migration and the epithelial-mesenchymal transition (EMT) process by activating the Wnt signaling pathway, in turn inducing hepatocarcinogenesis (16,17). It has been reported that such lncRNAs were associated with HBx; however, the precise role of HBx-related lncRNAs in primary liver cancer remains unknown. In addition, improved insight as to how HBx regulates the expression of lncRNAs requires further investigation.

lncRNA-activated by transforming growth factor (TGF)- β (lncRNA-ATB) is a recently identified oncogenic lncRNA, which is highly expressed in primary liver cancer tissues and several liver cancer cell lines. Yuan *et al* (18) revealed that lncRNA-ATB, induced by exogenous TGF- β , could promote the invasion-metastasis cascade by upregulating zinc finger E-box binding homeobox 1 and activating signal transducer and activator of transcription 3 (STAT3) signaling. Whether lncRNA-ATB is involved in the development of HBV-associated liver cancer and the exact mechanisms of lncRNA-ATB upregulation have not been elucidated.

Autophagy is an essential physiological process in the eukaryotic cell that regulates the metabolic situation in cells (19). Autophagy removes excess or self-damaged organelles, nucleic acids, macromolecular proteins and few degradation products to facilitate cell recycling, which in turn maintains intracellular homeostasis (20,21). Li *et al* (22) revealed that autophagy-induced TGF- β signaling contributed to the EMT and invasion of liver cancer cells; whether autophagy-induced TGF- β signaling occurs in HBV-related liver cancer is unknown.

The aim of the present study was to clarify the effects of lncRNA-ATB on the development of HBV-related liver cancer and its regulatory mechanism mediated by HBx. The results demonstrated that high levels of lncRNA-ATB were positively associated with hepatitis B surface antigen and tumor-node-metastasis (TNM) stage. Cell experiments revealed that HBx promoted the cell invasion and migration of liver cancer by upregulating lncRNA-ATB expression. Furthermore, TGF- β was significantly overexpressed by HBx-induced autophagy and activated lncRNA-ATB. These results suggested that the oncogenic effects of lncRNA-ATB could be mediated by HBx protein and may provide novel insight into the role of lncRNAs in the progression of HBV-related liver cancer.

Materials and methods

Cell culture and liver cancer tissues. The normal human hepatocyte cell line L02 and human liver cancer cell line HepG2 were purchased from the Cell Bank of the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The two cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 100 IU/ml penicillin and 100 μ g/ml streptomycin in 5% CO₂ at 37°C, for subsequent experimental research.

A total of 26 HBV-related liver cancer and non-HBV infected liver cancer tissues were obtained from patients who underwent radical resections for primary liver cancer in Nanjing Drum Tower Hospital (Nanjing University, Nanjing, China) during April 2014 to March 2015. These liver cancer samples were from 17 males and 9 females (37-80 years old). All patients were diagnosed by histopathological examination and the detection of HBV markers. In the subsequent experiments, all patients were separated into two groups based on lncRNA-ATB expression, and the cut-off was the 50th percentile. Tissues following the resection procedures were immediately placed in liquid nitrogen, then stored at -80°C. The present study was approved by the Ethics Committee of Nanjing Drum Tower Hospital and written informed consent was provided by all patients.

Lentivirus production and construction of stable cell lines with HBx overexpression. For the development of lentiviral vectors expressing the HBx gene, HBx cDNA was amplified by polymerase chain reaction (PCR) and subcloned into the lentiviral vector pHLV-CMVIE-ZsGreen-T2A-Puro using the one step directed cloning kit (Hanbio Biotechnology Co., Ltd., Shanghai, China); empty vector and non-transfected cells served as the control. The restriction enzyme used for HBx cDNA and vector were *EcoRI/XhoI* and *XbaI/BamHI*, respectively (Takara Biotechnology Co., Ltd., Dalian, China). The PCR procedure includes: Pre-denaturation at 95°C for 5 min, 95°C for 45 sec, 60°C for 45 sec, 72°C for 1 min (35 cycles) and finally the last cycle 72°C for 10 min, using an ABI StepOne Plus system (Applied Biosystems; Thermo Fisher Scientific, Inc.). To produce lentiviruses containing the HBx gene, the present study transfected 293 cells (Hanbio Biotechnology Co., Ltd.) with the resulting vector described above, pSPAX2 and pMD2G (Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine 2000™ (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols (23). Infectious lentiviruses were harvested at 48 and 72 h post-transfection and filtered through 0.45 μ m polyvinylidene fluoride (PVDF) filters, then centrifuged at 4°C, 72,000 x g/min for 120 min in a 40 ml ultracentrifuge tube, dissolved in DMEM and stored in liquid nitrogen. These lentiviruses were termed LV-control and LV-HBx, respectively. The two lentiviral vectors encoded green fluorescent protein.

The respective recombinant lentiviruses were added to HepG2 cells plated in a 6-well plate with a MOI of 20 to obtain cell lines stably expressing HBx. The supernatant was replaced with complete culture media (DMEM with 10% fetal bovine serum) following 24 h. Cells stably expressing HBx were

screened out following the addition of 2 μ g/ml puromycin into the media. Reverse transcription-quantitative PCR (RT-qPCR) and western blotting were performed to confirm the expression of HBx in the infected cells following infection.

Construction of the starvation-induced autophagy model.

The induction of cell autophagy was conducted according to Klionsky *et al* (24). The cell medium of each group was refreshed when the 6-well plate was covered with cells at 85% confluence; the control cells were cultured with fresh DMEM, and the experimental cells were washed three times with PBS to remove the residual medium and then cultured with 1X Earle's balanced salt solution (EBSS; Invitrogen; Thermo Fisher Scientific, Inc.) instead of DMEM, to generate a starvation environment with sugar and amino acid deficiency, and consequently induce autophagy. Cells were then incubated at 37°C and 5% CO₂ for 8 h and the optimal duration for the induction of autophagy selected in this experiment was 4 h post-starvation. Following the successful establishment of the model, total RNA and protein in each group of cells were extracted for subsequent analyses.

RNA interference and transfection. Small interfering (si)RNAs against lncRNA-ATB and TGF- β (40 nmol/l) were transfected into HepG2 and HBx-HepG2 cells respectively, which were plated in a 6-well plate at a density of 50%, using Lipofectamine 3000 according to manufacturer's instructions. Scramble-control siRNA was used as the control, which was transfected into the cells in the same manner as si-ATB and si-TGF- β transfection. The catalogue numbers of control/si-TGF- β /si-ATB are siN05815122147, siB09212165524 and siB160908101333, respectively (Guangzhou RiboBio Co., Ltd.). RT-qPCR confirmed the knockdown effect in HepG2 and HBx-HepG2 cells at 48 h post-transfection.

RNA extraction and RT-qPCR analysis. Total RNA was isolated from cultured cells or tumor tissues using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The first-strand cDNA was synthesized using PrimeScript[™] RT Master Mix (Takara Biotechnology Co., Ltd.); the conditions for RT were: 37°C for 20 min and then heated at 85°C for 15 sec. cDNAs could be stored at -20°C. qPCR was conducted with mixed cDNAs, gene primers and SYBR Green PCR master mix (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocols, while the RNA expression levels were measured using an ABI StepOne Plus system (Applied Biosystems; Thermo Fisher Scientific, Inc.). QPCR reactions were performed in triplicate, while β -actin and 18S rRNA were selected as the internal controls. The primers utilized for qPCR were as follows: lncRNA-ATB forward, 5'-TCTGGCTGAGGCTGGTTGAC-3', reverse, 5'-ATCTCTGGGTGCTGGTGAAGG-3'; HBx forward, 5'-CCCGTCTGTGCCTTCATC-3', reverse, 5'-GTATGCCTC-AAGTCCGGTCG-3'; β -actin forward, 5'-GGGAAATCGTGCCTGACATTAAG-3', reverse, 5'-TGTGTTGAGCGATCAGGTCTTTG-3'; Beclin-1 forward, 5'-CAGGAGAGACCCAGGAGGAA-3', reverse, 5'-GCTGTTGGCACTTTCTGTGG3'; microtubule-associated proteins 1A/1B light chain 3B (LC3b) forward, 5'-CCGCACCTTCGAACAAAGAG-3', reverse, 5'-TTGAGCTGTAAGCGCCTTC-T-3'; sequestosome 1 (SQSTM1) forward, 5'-TCT

GGCTGAGGCTGGTTGAC-3', reverse, 5'-ATCT-CTGGGTGCTGGTGAAGG-3' and TGF- β forward, 5'-CACCATAAA GACAGGAACCTG-3', reverse, 5'-GGAGGTGCCATCAATACCTGC3'. The relative expression of RNAs was calculated using the comparative 2^{- $\Delta\Delta$ C_q} method (25,26).

Western blot analysis. Lysis buffer was used for the extraction of total proteins of cells and tissues, which were quantified using a Bicinchoninic Acid kit (Nanjing KeyGEN Biotech Co., Ltd., Nanjing, China). Equal quantities of proteins (4 mg/ml) were separated by SDS-PAGE (with 30% acrylamide), then transferred onto PVDF membranes. Following incubation with antibodies (1:1,000) specific for HBx (ab39716; Abcam, Cambridge, UK), LC3b (cat. no. 3868; Cell Signaling Technology, Inc., Danvers, MA, USA), p62 (cat. no. 23214; Cell Signaling Technology, Inc.), Beclin-1 (cat. no. 4122; Cell Signaling Technology, Inc.), TGF- β (ab186838; Abcam) and β -actin (ab179467; Abcam) for 12 h at 4°C; then the blots were incubated with goat anti-rabbit (for HBx, LC3b and p62) or anti-mouse (for TGF- β , β -actin and Beclin-1) IgG-horseradish peroxidase-conjugated antibodies (1:5,000; Nanjing KeyGEN Biotech Co., Ltd.) for 2 h at room temperature. The proteins were semi-quantified by chemiluminescence method (Tanon Science & Technology Co., Ltd., Shanghai, China) and analyzed using ImageJ software (Version 1.5.1; National Institutes of Health, Bethesda, MD, USA).

In vitro cell invasion and migration assays. A Transwell assay was used to determine cell invasion as described previously (27). Cells ($\sim 5 \times 10^4$) were resuspended in 200 μ l serum-free medium and added to the upper layer of the Transwell chamber, while 500 μ l of complete medium was added into the lower chamber. Following 48 h of culture at 37°C, the medium was discarded and noninvaded cells were gently removed with a cotton swab and rinsed three times with PBS. The remaining cells were then fixed using 3.7% paraformaldehyde at room temperature for 20 min. The membranes were stained with 0.1% crystal violet for 30 min at room temperature, and cell invasive ability was analyzed by counting the number of stained cells in random fields of view under a 400-fold inverted biological microscope.

Cell migration ability was evaluated using a scratch assay. A 200- μ l pipette tip was used to scratch a straight wound in cells seeded in 6-well plates and then cultured in serum-free medium for 24 h at 37°C. Images of the wound width were then captured at 0 and 24 h and compared under a 5-fold inverted biological microscope. All experiments were independently repeated in triplicate.

Statistical analysis. SPSS 19.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Each group of experiments was repeated at least three times, and data are expressed as the mean \pm standard deviation. The χ^2 test was used to assess the association between lncRNA-ATB expression and the clinical characteristics of patients. A two-tailed Student's t-test was used for the comparison of independent variables. One-way analysis of variance was used for the comparison of multiple groups, followed by a Student-Newman-Keuls post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

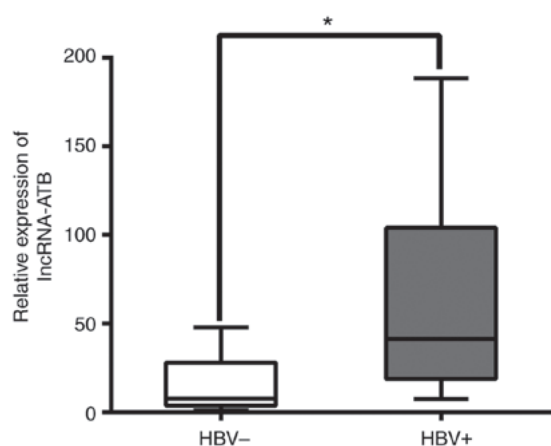


Figure 1. lncRNA-ATB is highly expressed in HBV-associated liver cancer. Reverse transcription-quantitative polymerase chain reaction analysis of lncRNA-ATB expression in HBV-related liver cancer tissues (n=13) and non-HBV infected liver cancer tissues (n=13). *P<0.05 vs. non-HBV infected liver cancer group. lncRNA-ATB, long noncoding RNA-activated by transforming growth factor β ; HBV, Hepatitis B virus.

Results

Expression of lncRNA-ATB in liver cancer and its association with clinicopathological characteristics. To clarify the role of lncRNA-ATB in primary liver cancer and whether HBx regulated lncRNA-ATB, the present study selected 26 tumor tissues from patients with radical hepatectomy and detected the expression of lncRNA-ATB by RT-qPCR. The association between lncRNA-ATB content and the clinicopathological characteristics of liver cancer, including the status of HBV infection, was then evaluated. As presented in Fig. 1, lncRNA-ATB was significantly associated with HBV. In addition, a significant association was detected between more advanced TNM stage and a higher expression levels of lncRNA-ATB, irrespective of patient age, gender, tumor size, liver cirrhosis and histological differentiation (Table I). These results suggested that increased expression of lncRNA-ATB in primary liver cancer may be associated with HBV infection and advanced tumor development.

HBx upregulates the expression of lncRNA-ATB in liver cancer cells. Previous studies have revealed that lncRNA-ATB can promote the invasion and migration of various tumor cells including primary liver cancer by activating the EMT and STAT3 pathways, while lncRNA-ATB could induced by TGF- β (18,28-30). However, whether abnormal expression of lncRNA-ATB is associated with the invasive and migration abilities of HBV-associated liver cancer cells remains unknown. To clarify this mechanism, the present study constructed an HBx lentivirus transfected HepG2 cell line (HBx-HepG2) which stably expressed HBx protein. The results of western blotting and RT-qPCR revealed that the levels of HBx protein and mRNA were markedly increased in HepG2 cells following transfection, while the expression of HBx in the blank control and empty plasmid groups (con-HepG2) was notable reduced (Fig. 2A and B).

To further investigate the effects of HBx on lncRNA-ATB in HBx-HepG2 cells, the present study compared the expression of

Table I. Association between lncRNA-ATB expression and clinical characteristics in patients with liver cancer (n=26).

Factors	lncRNA-ATB ^a		P-value ^b
	Low	High	
All cases	13	13	
Age			
≤55	6	8	0.430
>55	7	5	
Gender			
Male	10	8	0.394
Female	3	5	
HBsAg			
Positive	4	12	0.001
Negative	9	1	
Liver cirrhosis			
With	5	9	0.113
Without	8	4	
Tumor size, cm			
≤5	5	7	0.430
>5	8	6	
Histological differentiation			
Well	5	3	0.685
Moderate	7	9	
Poor	1	1	
TNM stage			
I + II	9	4	0.017
III + IV	2	11	

^aThe median lncRNA-ATB expression level was used as the cutoff. Low lncRNA-ATB expression in each of the 13 patients was defined as values below the 50th percentile. High lncRNA-ATB expression in 13 patients was defined as values at or above the 50th percentile.

^b χ^2 test was used to analyze the association between the expression levels of lncRNA-ATB and clinical characteristics of patients with liver cancer. lncRNA-ATB, long noncoding RNA-activated by transforming growth factor β .

lncRNA-ATB in L02, HepG2, con-HepG2 and HBx-HepG2 cell lines. As presented in Fig. 2C, the content of lncRNA-ATB in HBx-HepG2 cells was significantly increased following transfection with HBx lentivirus compared with HepG2 cells. The results were similar in HepG2 and con-HepG2 cells. In addition, the levels of TGF- β mRNA expression, which has been reported to be an inducer of lncRNA-ATB expression, were evaluated; a significant increase in HBx-HepG2 cells was observed compared with HepG2 cells (Fig. 2D). These results suggested that HBx may promote the expression of lncRNA-ATB in HepG2 cells by upregulating the expression of TGF- β .

HBx-induced autophagy significantly increases the expression of TGF- β and lncRNA-ATB. As aforementioned, alterations in cell behaviors, including metabolism, secretion or degradation of functional proteins, is one of the most critical underlying

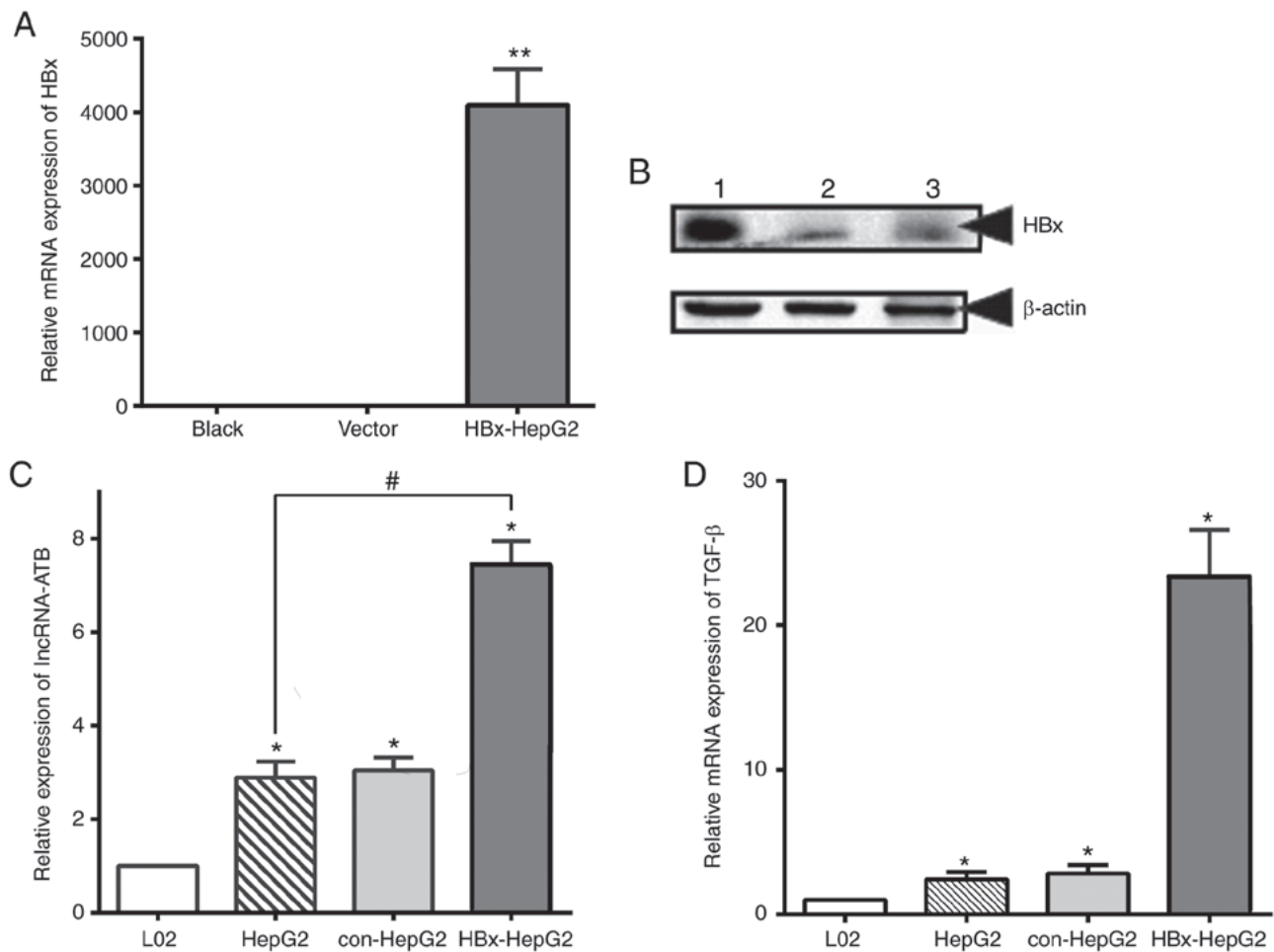


Figure 2. HBx upregulates the expression of lncRNA-ATB and TGF- β in HepG2 cells. (A) RT-qPCR analysis and (B) western blotting analysis of HBx expression in HepG2 cells transfected with lentivirus. Lane 1, HBx lentivirus transfected cells; lane 2, control vector and lane 3, blank. (C) RT-qPCR analysis of lncRNA-ATB in L02, HepG2, con-HepG2 and HBx-HepG2 cells. (D) RT-qPCR analysis of TGF- β in HepG2 and HBx-HepG2 cells. 18S rRNA was used as the internal control. *, $P < 0.05$, ** $P < 0.01$. HBx, Hepatitis B virus X protein; lncRNA-ATB, long noncoding RNA-activated by transforming growth factor β ; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; con-HepG2, empty plasmid group; TGF- β , transforming growth factor- β .

mechanisms as to how HBx leads to the development of primary liver cancer (5,31). In recent years, autophagy has been reported as an essential mechanism for regulating the intracellular environment (20,32). Whether HBx can affect the expression of intracellular lncRNA-ATB by mediating the autophagy of liver cancer cells is unclear. To investigate this, the present study detected the level of autophagy in HepG2 and HBx-HepG2 cells. As presented in Fig. 3A and B, the expression of autophagy-associated markers SQSTM-1 and LC3 in HBx-HepG2 cells were significantly increased following HBx transfection, while Beclin-1, which reflects the successful synthesis of autophagic precursors, exhibited a significant decrease compared with in HepG2 cells. HBx-HepG2 cells exhibited a marked increase in the expression of p62 compared with in HepG2 cells. These results indicated that HBx could promote autophagy in HepG2.

To further clarify whether lncRNA-ATB expression levels in HBx-HepG2 are associated with high levels of autophagy, the present study designed starvation-induced autophagy models using EBSS, which has been widely used for the induction of autophagy (33,34). Alterations in autophagy-associated proteins in each group were detected once cells were cultured with serum-free essential culture medium EBSS for 4 h. The expression levels of Beclin-1 and LC3 were significantly

increased in HepG2 and HBx-HepG2 cells following 4 h of starvation (Fig. 3C-E). The increase in LC3 II/LC3 I indicated an increase in autophagy, while the upregulated Beclin-1 suggests that autophagy may occur rapidly and autophagic precursor protein may be synthesized in marked quantities.

Following the successful induction of autophagy, the content of lncRNA-ATB in HepG2 and HBx-HepG2 cells increased significantly at 4 h compared with 0 h of starvation (Fig. 4A). Analysis of the lncRNA-ATB inducer, TGF- β , revealed that the mRNA and protein expression levels of TGF- β were upregulated in the two cell lines at 4 h of starvation than at 0 h (Fig. 4B-D). These results indicated that starvation-induced autophagy could significantly increase the levels of lncRNA-ATB and TGF- β in HepG2 cells transfected with HBx.

Knockdown of lncRNA-ATB/TGF- β could inhibit the level of autophagy in HepG2 cells. The aforementioned findings indicated an association between autophagy and TGF- β /lncRNA-ATB expression in HBx-HepG2 cells. To further verify this association, the present study employed siRNAs against lncRNA-ATB and TGF- β to observe their effects on cell autophagy following knockdown of lncRNA-ATB/TGF- β . The results of RT-qPCR

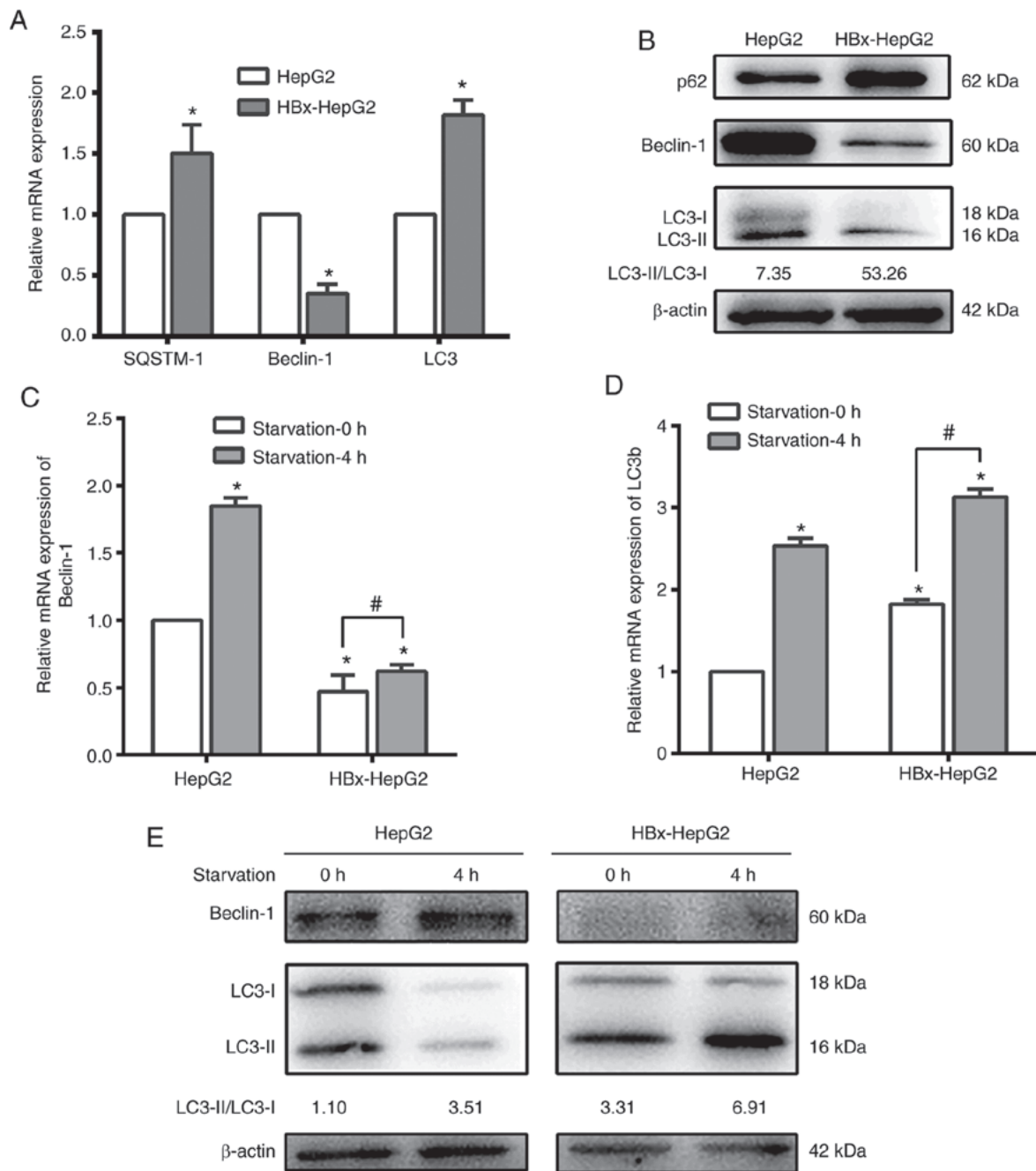


Figure 3. HBx induces autophagy in HepG2 cells. (A and B) Comparison of the expression of autophagy-associated markers, SQSTM-1, Beclin-1 and LC3, in HepG2 and HBx-HepG2 cells, as measured by reverse transcription-quantitative polymerase chain reaction and western blotting. (C-E) Alterations in Beclin-1 and LC3 expression in the two cell lines, following culture with serum-free basic culture medium Earle's balanced salt solution for 4 h. Western blotting analysis revealed the ratio of LC3-II/LC3-I was significantly elevated following cell starvation. * $P < 0.05$ vs. HepG2 (starvation-0 h); # $P < 0.05$. HBx, Hepatitis B virus X protein; LC3b, microtubule-associated proteins 1A/1B light chain 3B; SQSTM-1, sequestosome 1.

demonstrated that the expression of target RNA in cells was significantly inhibited following siRNA-ATB or siRNA-TGF- β transfection in HepG2 and HBx-HepG2 cells compared with the control, indicating successful transfection (Fig. 5A and B). In addition, the expression of lncRNA-ATB was significantly decreased following knockdown of TGF- β compared with the control (Fig. 5C). Conversely, the expression levels of TGF- β were markedly altered following the knockdown of lncRNA-ATB (data not shown), which suggested that TGF- β also regulates the expression of lncRNA-ATB in HBx-HepG2 cells.

Further investigation demonstrated that LC3 expression was suppressed following the knockdown of lncRNA-ATB or TGF- β in HepG2 cells; however, these observations were not noted in HBx-HepG2 cells (Fig. 5D-G). These results demonstrated that, at least in part, autophagy was inhibited in the case of lncRNA-ATB knockdown in liver cancer cells, while the expression of HBx could partially alleviate this inhibition.

Starvation-induced autophagy upregulates the expression of lncRNA-ATB following TGF- β knockdown. To further detect the association between HBx-induced autophagy and

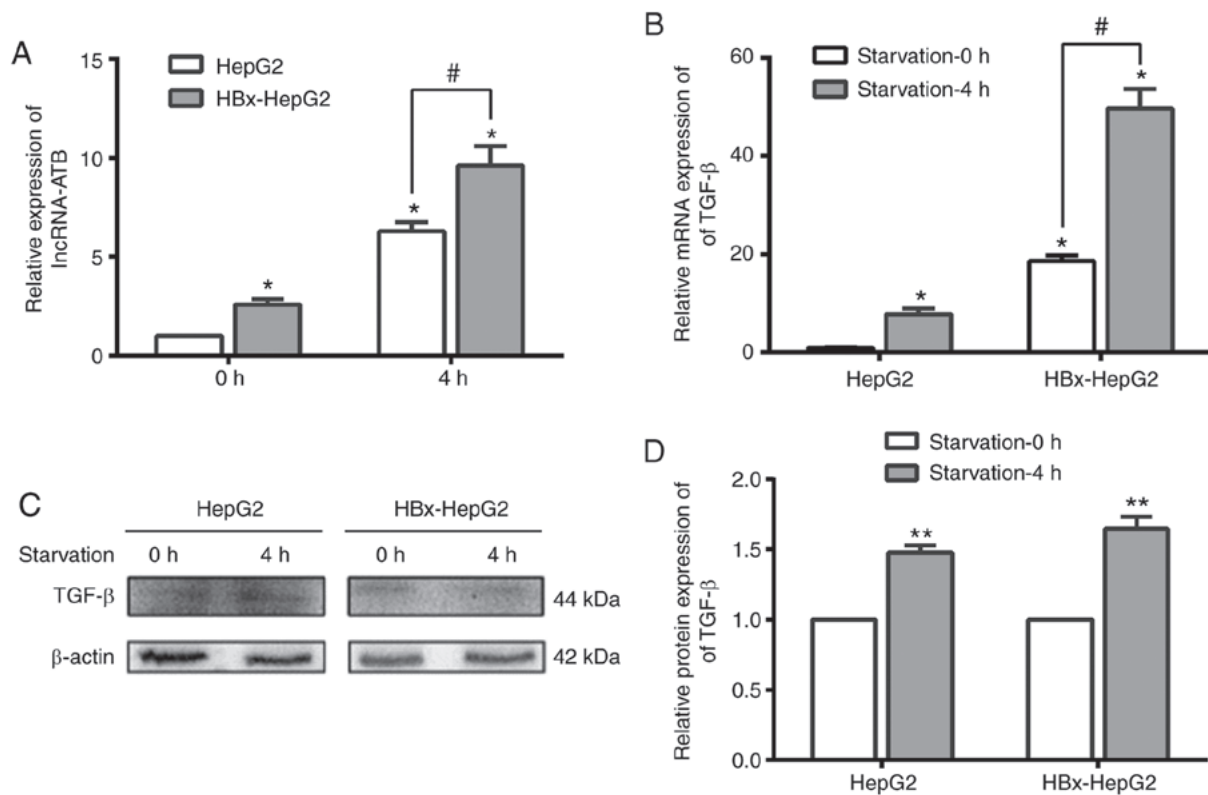


Figure 4. HBx-induced autophagy upregulates the expression of lncRNA-ATB and TGF-β. The expression of (A) lncRNA-ATB and (B) TGF-β mRNA in HepG2 and HBx-HepG2 cells following starvation-induced autophagy. (C and D) Western blotting analysis of TGF-β in the two cell lines, which supported the findings from reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. HepG2 (starvation-0 h); #P<0.05; **P<0.01. HBx, Hepatitis B virus X protein; lncRNA-ATB, long noncoding RNA-activated by transforming growth factor; TGF, transforming growth factor β.

lncRNA-ATB, the present study determined lncRNA-ATB expression in starvation-induced autophagy and the suppression of TGF-β in HepG2 and HBx-HepG2 cells. The mRNA levels of the autophagy-associated protein LC3b were significantly increased following starvation for 4 h, while such an increase was observed in HBx-HepG2 and HepG2 cells transfected with si-TGF-β (Fig. 6A and B). Combined with previous experiments, this suggested that HBx-induced autophagy could promote the expression of TGF-β but was not dependent on it. The present study then investigated the expression levels of lncRNA-ATB under these conditions. As presented in Fig. 6C and D, si-TGF-β significantly inhibited the expression of lncRNA-ATB compared with the control at 0 h, but was significantly upregulated by starvation-induced autophagy in HepG2 and HBx-HepG2 cells. Thus, it was concluded that HBx-induced autophagy led to the abnormal expression of TGF-β, and upregulated that of lncRNA-ATB in liver cancer cells.

Effects of lncRNA-ATB on the invasion and migration abilities of HBx-HepG2 cell lines. It has been reported that the invasion and migration abilities of HBV-associated liver cancer cells were greater than normal hepatocyte cell lines (35,36); however, whether lncRNAs participate in the invasion and migration of HBV-associated primary liver cancer is unclear. In the present study, stably transfected HBx-vector significantly promoted cell invasion and migration compared with HepG2 cells (Fig. 7A-D). A scratch-wound assay was then conducted to investigate whether TGF-β or lncRNA-ATB affected

the migration ability of HBx-HepG2 cells. As presented in Fig. 7E-H, the migration ability of HBx-HepG2 cells was significantly inhibited when TGF-β was suppressed compared with the control group, which was not apparent in HepG2 cells. In addition, such inhibition of migration was significant in the two types of cells transfected with siRNA-ATB (Fig. 7I-L). Therefore, it was concluded that the invasion and migration of HBx-HepG2 may be associated with the expression of lncRNA-ATB. Abnormal expression of lncRNA-ATB may be an underlying mechanism of enhanced invasion and migration of HBV-associated liver cancer cells.

Discussion

It has been reported that ~50% of primary liver cancer cases are caused by HBV infection in China (37). HBV-associated liver cancer is more prone to invasion and distant metastasis than cancer without HBV infection, but the mechanism of HBV in primary liver cancer development requires further investigation (17,38). As HBV-related liver cancer is one of the most common subtypes of liver cancer in China, the HBx protein encoded by X gene has been reported to be the primary pathogenic factor of primary liver cancer (31). Therefore, it is necessary to improve the early diagnosis rate of HBV-related liver cancer, in order to identify potential tumor markers with high sensitivity and specificity. Following the discovery of lncRNA and in-depth study of its function, increasing evidence has suggested that lncRNAs may be involved in the invasion and metastasis of liver cancer (12,13,39); however,

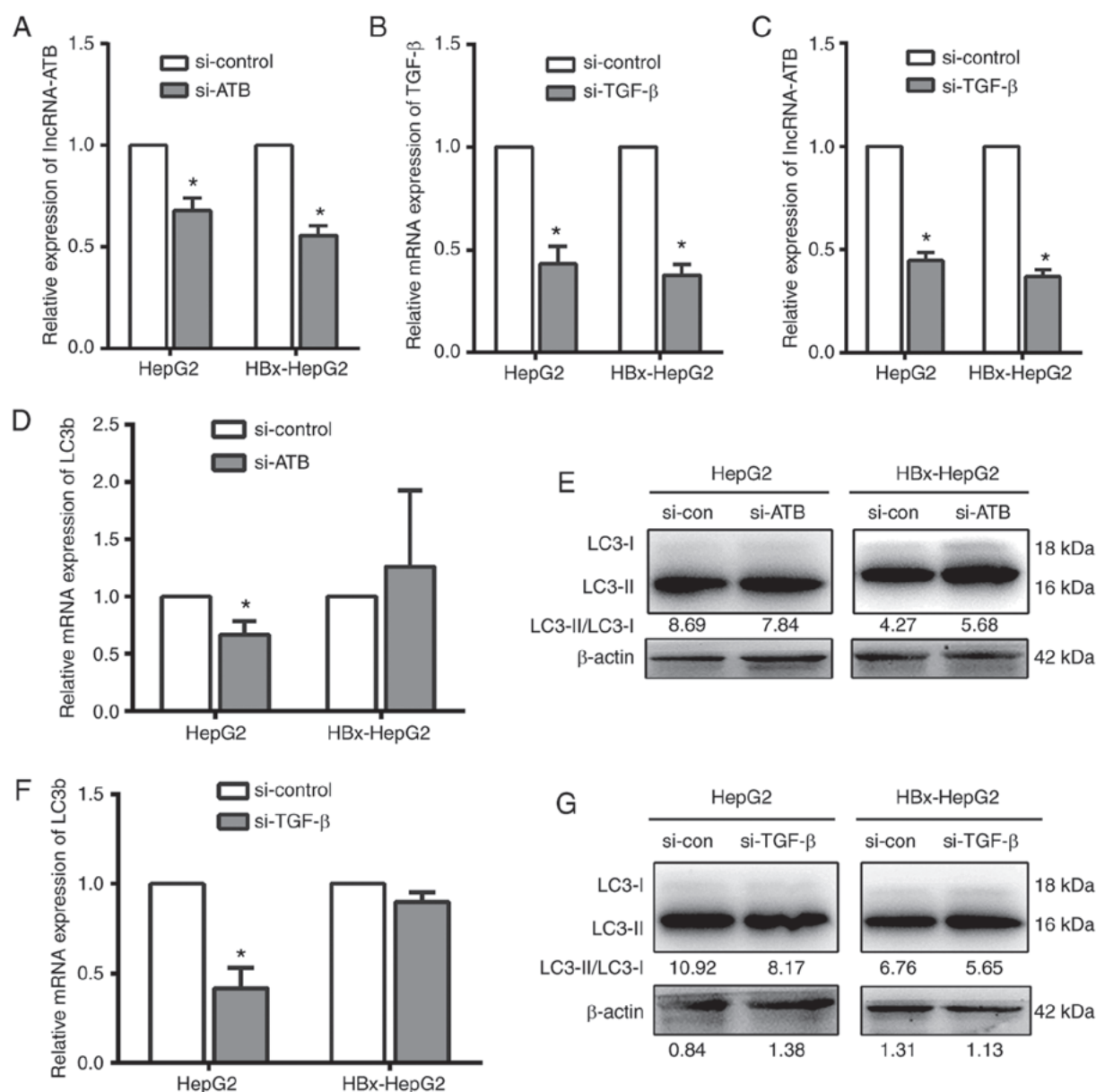


Figure 5. Knockdown of lncRNA-ATB or TGF- β with siRNAs inhibits autophagy in HepG2 cells. Reverse transcription-quantitative polymerase chain reaction analysis of (A) lncRNA-ATB and (B) TGF- β mRNA in HepG2 and HBx-HepG2 cells transfected with si-lncRNA-ATB, si-TGF- β or negative control. (C) Evaluation of lncRNA-ATB in HepG2 and HBx-HepG2 cells transfected with si-TGF- β or negative control. Alterations in autophagic activity in HepG2 and HBx-HepG2 cells following (D and E) si-lncRNA-ATB or (F and G) si-TGF- β transfection, as indicated by LC3 mRNA expression and the ratio of LC3-II/LC3-I. * $P < 0.05$ vs. si-control. HBx, Hepatitis B virus X protein; LC3, microtubule-associated proteins 1A/1B light chain 3; lncRNA-ATB, long noncoding RNA-activated by transforming growth factor β ; TGF, transforming growth factor; si, small interfering RNA.

whether the abnormal expression of lncRNAs is associated with the HBV infection in liver cancer remains unknown. lncRNA-ATB is a novel lncRNA associated with liver cancer that can be overexpressed via the addition of exogenous TGF- β ; lncRNA-ATB functions as a competing endogenous RNA by activating the EMT pathway, and promotes the invasion and distant metastasis of tumor cells (18,30). From these findings, the present study hypothesized that HBx could upregulate the expression of lncRNA-ATB, while overexpression of lncRNA-ATB may promote the invasion and migration of liver cancer cells. To the best of our knowledge, the present study is the first to detect the expression of lncRNA-ATB in primary liver cancer tissues with or without HBV infection. The expression levels of lncRNA-ATB in HBV-related liver

cancer were significantly increased than in patients without HBV infection; tissues with higher lncRNA-ATB levels were linked with advanced TNM stage, which indicated that HBV could upregulate lncRNA-ATB, a pathogenic factor associated with the development of liver cancer.

However, whether the role of HBx protein in the occurrence of liver cancer is associated with the abnormal expression of lncRNA-ATB remains unknown. In the present study, HBx-HepG2 cells, which stably expressed HBx was constructed and the expression of lncRNA-ATB and TGF- β in HepG2 and HBx-HepG2 cells was compared. The results revealed that HBx upregulated the expression of lncRNA-ATB and TGF- β . Based on these observations, it was proposed that this phenomenon may be due to the effects of autophagy in HepG2 cells. Previous

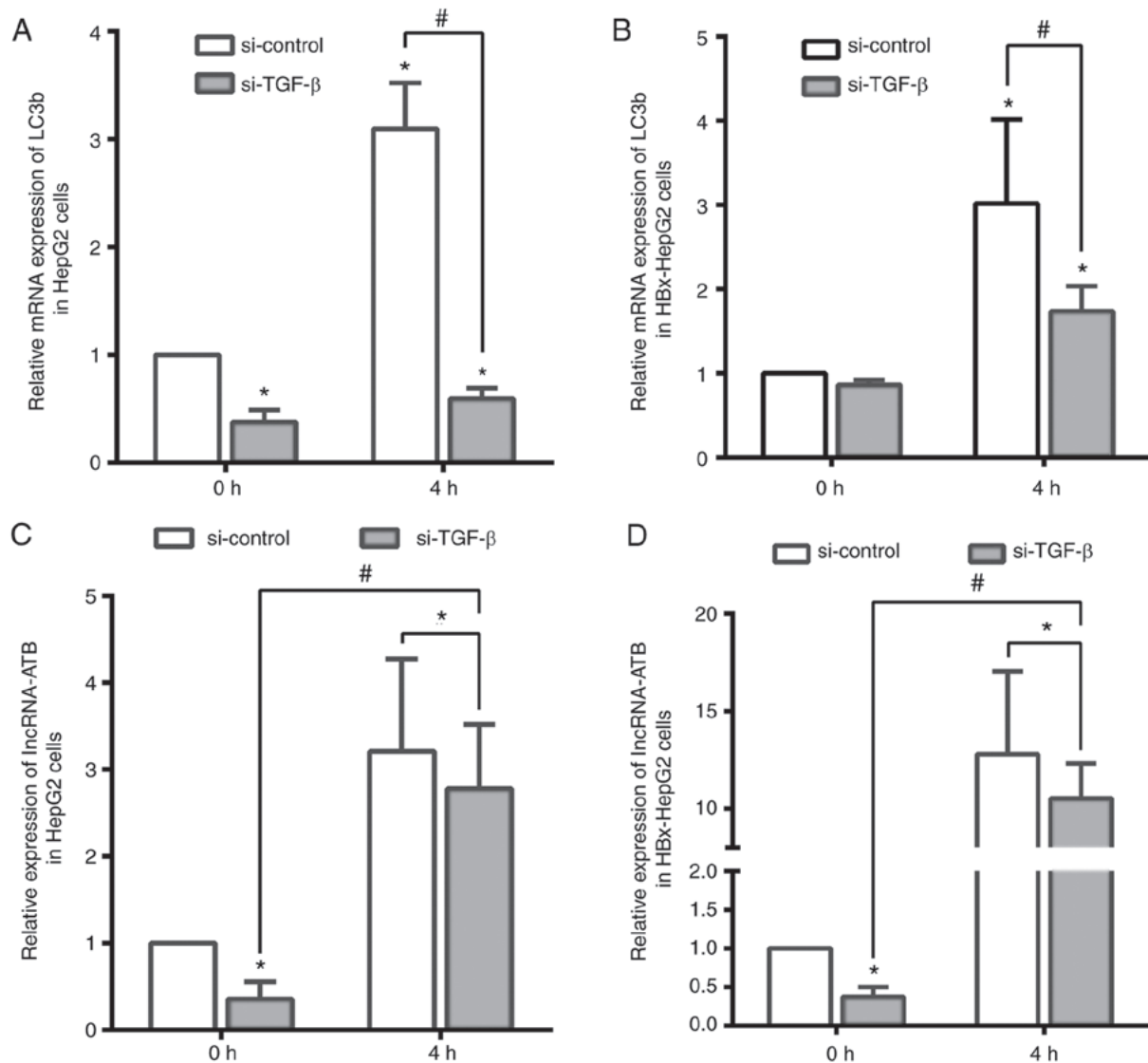


Figure 6. Starvation-induced autophagy attenuates the suppression of lncRNA-ATB following TGF- β knockdown. Autophagic activity under EBSS-induced starvation was measured in (A) HepG2 and (B) HBx-HepG2 cells, which were transfected with si-TGF- β or negative control. LC3b mRNA expression was used as the autophagy indicator. Alterations in lncRNA-ATB expression in (C) HepG2 and (D) HBx-HepG2 cells under EBSS-induced starvation. The two cell lines were transfected with si-TGF- β or negative control. *P<0.05 vs. si-control (0 h); #P<0.05. HBx, Hepatitis B virus X protein; lncRNA-ATB, long noncoding RNA-activated by transforming growth factor β ; EBSS, Earle's balanced salt solution; TGF, transforming growth factor; si, small interfering RNA.

studies have demonstrated that autophagy is an essential process in eukaryotes, that can affect or reflect the synthesis and degradation of RNA and proteins, including TGF- β (19,22,40). To confirm our hypothesis, the present study investigated the expression levels of the autophagy-associated proteins p62, Beclin-1 and LC3b in HBx-HepG2 and HepG2 cells. The results revealed that the expression of p62 and LC3b in HBx-HepG2 were significantly higher than in HepG2, suggesting that HBx may be associated with the induction of autophagy in HepG2. A model of starvation-induced autophagy was constructed to simulate the induction of autophagy by HBx, while the over-expression of lncRNA-ATB and TGF- β were also detected in cells with or without HBx overexpression at 4 h of starvation. Furthermore, it was demonstrated that knockdown of TGF- β downregulated lncRNA-ATB in HBx-HepG2 cells as determined by the RNA interference experiments under conditions of starvation. Of note, knockdown of TGF- β or lncRNA-ATB could inhibit autophagy in HepG2, but it did not affect autophagy

within HBx-HepG2 cells. This suggested that TGF- β may affect autophagy in liver cancer cells, but the induction of autophagy by HBx could compensate for this mechanism.

The present study also detected the effects of lncRNA-ATB on the invasive and migration abilities of HepG2 cells following transfection with HBx, to elucidate the role of lncRNA-ATB in the progression of HBV-associated liver cancer. The results revealed that the invasive and migration abilities of HBx-HepG2 cells were promoted than in HepG2 cells, which were further reduced following the knockdown of TGF- β or lncRNA-ATB. This indicated that the invasive and metastatic potential of HBV-related liver cancer may be associated with the upregulated expression of TGF- β and lncRNA-ATB in liver cancer cells.

In conclusion, the present study demonstrated that HBx could upregulate the expression of TGF- β by inducing autophagy. Upregulated TGF- β may further stimulate the expression of lncRNA-ATB in primary liver cancer, and

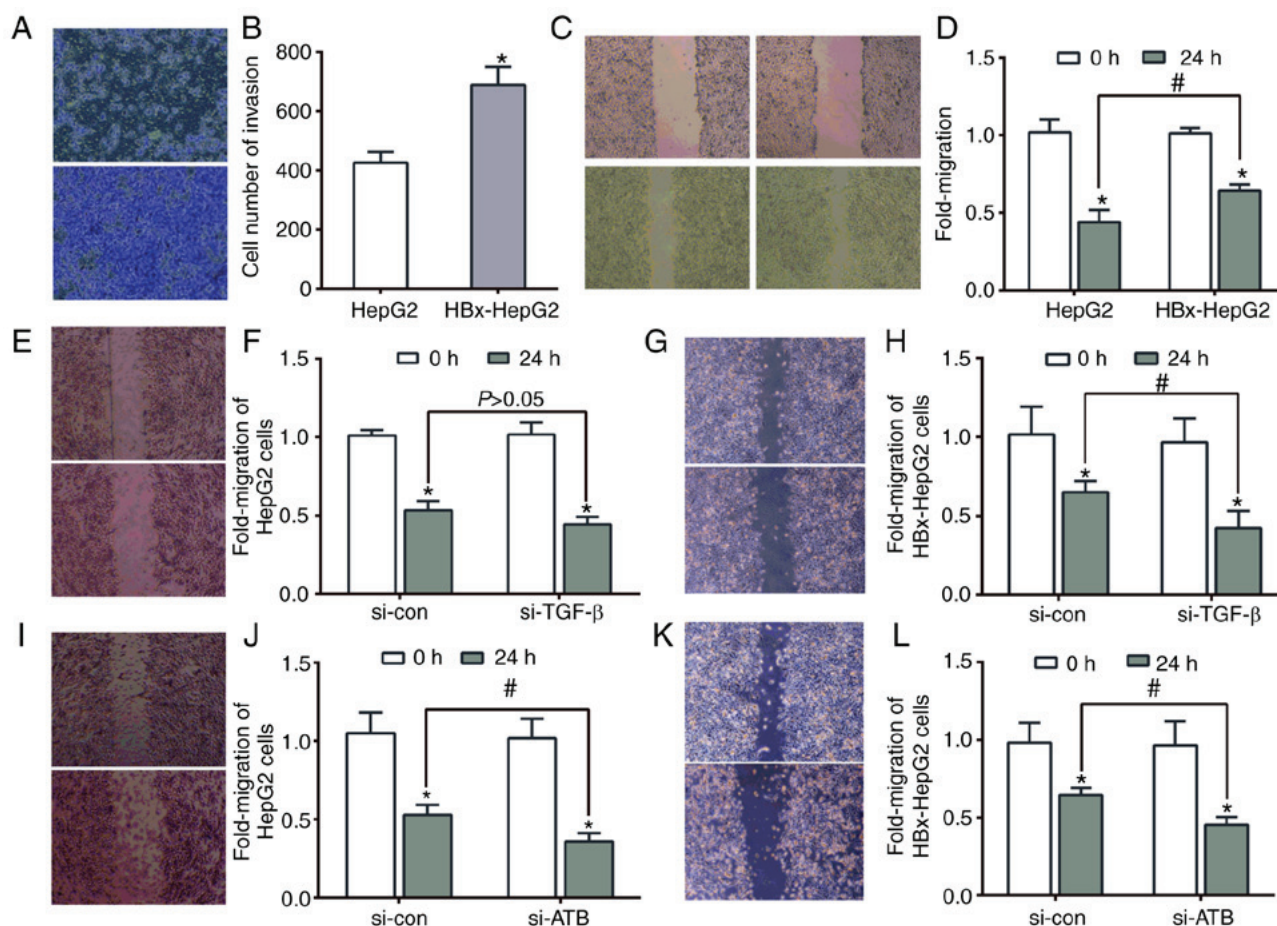


Figure 7. Suppression of long noncoding RNA-ATB or TGF- β inhibits the tumor-promoting effects of HBx in HepG2 cells. The invasive and migration abilities of HepG2 and HBx-HepG2 cells, measured by (A and B) Transwell and (C and D) scratch-wound assays. Transwell assay of (E and F) HepG2 cells and (G and H) HBx-HepG2 cells, following si-TGF- β and si-con transfection. Transwell assay of (I and J) HepG2 cells and (K and L) HBx-HepG2 cells, following si-ATB and si-con transfection. * $P < 0.05$ vs. HepG2 (0 h) or si-con (0 h); # $P < 0.05$. con, control; HBx, Hepatitis B virus X protein; ATB, activated by transforming growth factor β ; TGF, transforming growth factor; si, small interfering RNA.

finally promote the invasion and migration of liver cancer cells. These results may provide novel insight into the development of HBV-related liver cancer. lncRNA-ATB may be considered as a therapeutic target in the treatment of liver cancer; however, whether the altered invasion and migration abilities induced by lncRNA-ATB affects normal hepatocyte requires further investigation.

Acknowledgements

We thank Dr Jinglin Wang (Nanjing Drum Tower Hospital, Nanjing, China) for technical assistance.

Funding

The present study was supported by a grant from the National Natural Science Foundation of China (grant no. 81670566) and Jiangsu Province's Key Provincial Talents Program (grant no. ZDRCA2016066).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YHZ, JL, WZ and XLS made substantial contributions to the design of the present study. YHZ, JL and FJY performed the experiments. YHZ, JL and XLS wrote the manuscript. YZ, SW and YL were involved in data analysis and produced initial figure drafts. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Nanjing Drum Tower Hospital (Nanjing, China) and written informed consent was provided by all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J: Cancer statistics in China, 2015. *CA Cancer J Clin* 66: 115-132, 2016.
- Han LL, Lv Y, Guo H, Ruan ZP and Nan KJ: Implications of biomarkers in human hepatocellular carcinoma pathogenesis and therapy. *World J Gastroenterol* 20: 10249-10261, 2014.
- Maucourt-Boulch D, de Martel C, Franceschi S and Plummer M: Fraction and incidence of liver cancer attributable to hepatitis B and C viruses worldwide. *Int J Cancer* 142: 2471-2477, 2018.
- de Lope CR, Tremosini S, Forner A, Reig M and Bruix J: Management of HCC. *J Hepatol* 56 (Suppl 1): S75-S87, 2012.
- Tang H, Oishi N, Kaneko S and Murakami S: Molecular functions and biological roles of hepatitis B virus x protein. *Cancer Sci* 97: 977-983, 2006.
- Martin-Vilchez S, Lara-Pezzi E, Trapero-Marugán M, Moreno-Otero R and Sanz-Cameno P: The molecular and pathophysiological implications of hepatitis B X antigen in chronic hepatitis B virus infection. *Rev Med Virol* 21: 315-329, 2011.
- Ponting CP, Oliver PL and Reik W: Evolution and functions of long noncoding RNAs. *Cell* 136: 629-641, 2009.
- Wang KC and Chang HY: Molecular mechanisms of long noncoding RNAs. *Mol Cell* 43: 904-914, 2011.
- Ma L, Bajic VB and Zhang Z: On the classification of long non-coding RNAs. *RNA Biol* 10: 925-933, 2013.
- Caley DP, Pink RC, Trujillano D and Carter DR: Long noncoding RNAs, chromatin, and development. *ScientificWorldJournal* 10: 90-102, 2010.
- Yang F, Zhang L, Huo XS, Yuan JH, Xu D, Yuan SX, Zhu N, Zhou WP, Yang GS, Wang YZ, *et al*: Long noncoding RNA high expression in hepatocellular carcinoma facilitates tumor growth through enhancer of zeste homolog 2 in humans. *Hepatology* 54: 1679-1689, 2011.
- Wang J, Liu X, Wu H, Ni P, Gu Z, Qiao Y, Chen N, Sun F and Fan Q: CREB up-regulates long non-coding RNA, HULC expression through interaction with microRNA-372 in liver cancer. *Nucleic Acids Res* 38: 5366-5383, 2010.
- Fu WM, Zhu X, Wang WM, Lu YF, Hu BG, Wang H, Liang WC, Wang SS, Ko CH, Waye MM, *et al*: Hota1r mediates hepatocarcinogenesis through suppressing miRNA-218 expression and activating P14 and P16 signaling. *J Hepatol* 63: 886-895, 2015.
- Huang JF, Guo YJ, Zhao CX, Yuan SX, Wang Y, Tang GN, Zhou WP and Sun SH: Hepatitis B virus X protein (HBx)-related long noncoding RNA (lncRNA) down-regulated expression by HBx (Dreh) inhibits hepatocellular carcinoma metastasis by targeting the intermediate filament protein vimentin. *Hepatology* 57: 1882-1892, 2013.
- Du Y, Kong G, You X, Zhang S, Zhang T, Gao Y, Ye L and Zhang X: Elevation of highly up-regulated in liver cancer (HULC) by hepatitis B virus X protein promotes hepatoma cell proliferation via down-regulating p18. *J Biol Chem* 287: 26302-26311, 2012.
- Heikenwalder M and Protzer U: LINE(1)s of evidence in HBV-driven liver cancer. *Cell Host Microbe* 15: 249-250, 2014.
- Lau CC, Sun T, Ching AK, He M, Li JW, Wong AM, Co NN, Chan AW, Li PS, Lung RW, *et al*: Viral-human chimeric transcript predisposes risk to liver cancer development and progression. *Cancer Cell* 25: 335-349, 2014.
- Yuan JH, Yang F, Wang F, Ma JZ, Guo YJ, Tao QF, Liu F, Pan W, Wang TT, Zhou CC, *et al*: A long noncoding RNA activated by TGF- β promotes the invasion-metastasis cascade in hepatocellular carcinoma. *Cancer Cell* 25: 666-681, 2014.
- Levine B and Kroemer G: Autophagy in the pathogenesis of disease. *Cell* 132: 27-42, 2008.
- Ktistakis NT and Tooze SA: Digesting the expanding mechanisms of autophagy. *Trends Cell Biol* 26: 624-635, 2016.
- Ponpuak M, Mandell MA, Kimura T, Chauhan S, Cleyrat C and Deretic V: Secretory autophagy. *Curr Opin Cell Biol* 35: 106-116, 2015.
- Li J, Yang B, Zhou Q, Wu Y, Shang D, Guo Y, Song Z, Zheng Q and Xiong J: Autophagy promotes hepatocellular carcinoma cell invasion through activation of epithelial-mesenchymal transition. *Carcinogenesis* 34: 1343-1351, 2013.
- Stepanenko AA and Dmitrenko VV: HEK293 in cell biology and cancer research: Phenotype, karyotype, tumorigenicity, and stress-induced genome-phenotype evolution. *Gene* 569: 182-190, 2015.
- Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, Agholme L, Agnello M, Agostinis P, Aguirre-Ghiso JA, *et al*: Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* 8: 445-544, 2012.
- Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, *et al*: Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* 33: e179, 2005.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Hou Z, Xu X, Fu X, Tao S, Zhou J, Liu S and Tan D: HBx-related long non-coding RNA MALAT1 promotes cell metastasis via up-regulating LTP3 in hepatocellular carcinoma. *Am J Cancer Res* 7: 845-856, 2017.
- Ke L, Xu SB, Wang J, Jiang XL and Xu MQ: High expression of long non-coding RNA ATB indicates a poor prognosis and regulates cell proliferation and metastasis in non-small cell lung cancer. *Clin Transl Oncol* 19: 599-605, 2017.
- Saito T, Kurashige J, Nambara S, Komatsu H, Hirata H, Ueda M, Sakimura S, Uchi R, Takano Y, Shinden Y, *et al*: A long non-coding RNA activated by transforming growth factor- β is an independent prognostic marker of gastric cancer. *Ann Surg Oncol* 22 (Suppl 3): S915-S922, 2015.
- Xiong J, Liu Y, Jiang L, Zeng Y and Tang W: High expression of long non-coding RNA lncRNA-ATB is correlated with metastases and promotes cell migration and invasion in renal cell carcinoma. *Jpn J Clin Oncol* 46: 378-384, 2016.
- Feitelson MA, Reis HM, Tufan NL, Sun B, Pan J and Lian Z: Putative roles of hepatitis B x antigen in the pathogenesis of chronic liver disease. *Cancer Lett* 286: 69-79, 2009.
- Eitan E, Suire C, Zhang S and Mattson MP: Impact of lysosome status on extracellular vesicle content and release. *Ageing Res Rev* 32: 65-74, 2016.
- Tao H, Qian P, Lu J, Guo Y, Zhu H and Wang F: Autophagy inhibition enhances radiosensitivity of Eca-109 cells via the mitochondrial apoptosis pathway. *Int J Oncol* 52: 1853-1862, 2018.
- Barutcu SA, Girnius N, Vernia S and Davis RJ: Role of the MAPK/cJun NH2-terminal kinase signaling pathway in starvation-induced autophagy. *Autophagy* 14: 1586-1595, 2018.
- Ringelhan M and Protzer U: Oncogenic potential of hepatitis B virus encoded proteins. *Curr Opin Virol* 14: 109-115, 2015.
- Ding D, Lou X, Hua D, Yu W, Li L, Wang J, Gao F, Zhao N, Ren G, Li L and Lin B: Recurrent targeted genes of hepatitis B virus in the liver cancer genomes identified by a next-generation sequencing-based approach. *PLoS Genet* 8: e1003065, 2012.
- El-Serag HB: Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* 142: 1264-1273.e1, 2012.
- Chan HL and Sung JJ: Hepatocellular carcinoma and hepatitis B virus. *Semin Liver Dis* 26: 153-161, 2006.
- Gu H, Guo X, Zou L, Zhu H and Zhang J: Upregulation of microRNA-372 associates with tumor progression and prognosis in hepatocellular carcinoma. *Mol Cell Biochem* 375: 23-30, 2013.
- Lamb CA, Yoshimori T and Tooze SA: The autophagosome: Origins unknown, biogenesis complex. *Nat Rev Mol Cell Biol* 14: 759-774, 2013.