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Original Research Article

Effect of lncRNA pvt1 on HBV replication in HepG2.2.15 cells, and its possible mechanism of action

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Abstract

Purpose: *To investigate the influence of lncRNA pvt1 on hepatitis B virus replication in HepG2.2.15 cells and the underlying mechanism of action.*
Methods: *Concentrations of lncRNA pvt1 in HBV-positive liver cancer cell lines (HepAD38+, HepG2.2.15+, HepG2+, and HepaRG+), and 3 HBV-negative liver cancer cell lines were determined and compared. Differences in HBV DNA content, levels of HBsAg and HBeAg, and STAT3 axis signal pathway among groups were evaluated.*
Results: *LncRNA pvt1 levels in HBV-positive hepatoma cells were significantly higher than the corresponding expression levels in HBV-negative hepatoma cells. after si-pvt1 treatment in HepG2.2.15 cells, the 3.2K HBV DNA content increased significantly ($p < 0.05$), and the expression levels of HBsAg and HBeAg in HepG2.2.15 cells also significantly increased. In all groups, STAT3 protein level was comparable, but p-STAT3 protein expression in si-pvt1 group was significantly reduced, relative to those in Si NC and control groups ($p < 0.05$).*
Conclusion: *Levels of lncRNA pvt1 expression in HBV-positive and HBV-negative hepatoma cells differ significantly. Treatment with si-pvt1 decrease HBV DNA content in HepG2.2.15 cells through a mechanism that relates to the STAT3 axis signal pathway. This finding may be beneficial for follow-up treatment of HBV.*

Keywords: *Lnc-RNA pvt1, Hepatitis B virus, STAT3, mechanism*

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INTRODUCTION

The pathogen of hepatitis B (HBV) belongs to the family of hepatitis B DNA viruses. The clinical presentations of HBV infection are abnormal liver function and inflammation, as well as liver necrosis and fibrosis [1]. Chronic HBV infection patients are likely to develop severe hepatic disease, hepatocellular carcinoma, and cirrhosis. A recent study showed that about 292 million people worldwide are HBV carriers, and the annual death figure from HBV infection or its complications is nearly 900000 [2].

Usually, lncRNA which contains in excess of 200 base pairs, is crucial in various important and potential cardiovascular processes, including those related to cell proliferation, HBV

replication, and autophagy [3]. Recent findings indicate that lncRNA is important in HBV replication, and researchers are keenly interested in its impact on the occurrence, migration, diagnosis, and anti-resistance of CHB [4]. Recently, it was reported that lncRNA pvt1 was significantly up-regulated in HBV-positive HepG2 cells, and it enhanced the migratory and invasive potential of HBV-positive Hep3B cells but had no effect on HBV-negative HepG2 cells [5]. In contrast, the influence of lncRNA on HBV infection is not yet elucidated. This research was aimed at studying the influence of lncRNA on HBV proliferation. An in-depth investigation of the specific molecular mechanism of dry HBV replication may provide new strategies, new drug targets, and biomarkers for the clinical therapy of CHB.

EXPERIMENTAL

Cell culture

Hepatitis B virus (HBV)-positive liver cancer cell lines (HepG2+, HepARG+, HepAD38+, and HepG2.2.15) and the corresponding HBV-negative liver cancer cell lines were used in this study. Cell culture was carried out according to the conventional method in an incubator containing 5 % CO_2 at 37 ^oC.

Western blot assay

Total protein was extracted from the cells using RIPA buffer. The protein samples were kept at -70 °C prior to use. The proteins were subjected to SDS-PAGE, and sequentially transferred to PVDF films using the wet transfer method, sealed with 5 % fat-free milk powder for 2 h, and reacted with 1^o immunoglobulins (alkbh5, p-STAT3, and STAT3) for 12 h at 4 °C. Thereafter, the membrane was rinsed thrice in TBST, followed by incubation with 2^o immunoglobulin for 60 min at laboratory temperature for 1 h. Protein expression levels were determined using Odyssey-2 color infrared fluorescence imaging system.

Construction of plasma

A conventional cell transfection scheme was used to obtain lncRNA pvt1 siRNA plasma and lncRNA pvt1 siRNA NC plasma.

Southern blotting

After removing the culture medium, the cells were washed twice with PBS. Then, 500 µL of lysate was added, followed by a 15-min incubation at 37 °C. Then, the lysate was put in a 1.5-mL Eppendorf tube and concentrated for 5 min. It was incubated in a 37 °C water bath for 4 h after the addition of 5 µL of 1 mol/L $MgCl_2$ and 4 µL DNaseI. Then, 35 % PEG8000 solution was added, and the mixture was placed in an ice bath for 1 h. After spinning at 11000*g* at 4 °C for 5 min, 500 µL protease K digestive solution was added to the pellet to a final concentration of 500 µg/mL. Following thorough mixing, it was put in a 45 °C H_2O bath overnight. On the following day, the mixture was extracted with a 2:1 volume ratio of phenol and chloroform, and the nucleic acid was purified in 70 % isopropanol and rinsed in 70 % ethanol. Then, it was subjected to drying under laboratory conditions and dissolved in double-distilled H_2O (ddH_2O). The dissolved HBVDNA was separated using 0.9 % agarose gel electrophoresis and then subjected to Southern blotting. After alkaline degradation of the gel for 1 h, the DNA fragments on the gel were transferred to the nylon membrane in a 42 °C pre-hybrid solution for 1 h. Then, the digoxigenin-labeled HBV DNA probe was hybridized overnight at 42 °C. The film was washed, sealed, and washed again. The specific operations were carried out according to the instructions of the Southern blotting kit.

cccDNA extract

The HBV cccDNA cell culture medium was extracted with Hirt, rinsed two times using PBS, and subjected to 15-min lysis with 500 µL lysing buffer. Then, 125 µL of 2.5 mol/L KCl solution was put in the lysate. After 12-h shaking of the mixture at 4 °C, the mixture was centrifuged at 4 °C and 14000*g* for 20 min. The supernatant was extracted 3 times with an equivalent volume of phenol/chloroform, and the nucleic acid was purified with 70 % isopropanol and rinsed with 70 % ethanol. After drying, the nucleic acid was dissolved in 20 µL of ddH_2O. The sample was digested with T5 nuclear acid exonuclease (1000 IU/mL) for 1 h to remove non-closed circular DNA and then heated at 99 ° C for 5 min to inactivate the enzyme.

RT qPCR

TRIzol kit was employed for the extraction of total RNA of cells and RNA of liver tissues. Then, 1 microgram of RNA extract was converted to cDNA with cDNA synthesis kit. Fluorescence quantitative PCR was carried out with SYBR Green supermax in a reaction system comprised of 0.2 µL of 10 µM primer, 5 µL SYBR Green supermax, 3.6 µL of ddH_2O, and 1 µL of reverse transcription product. The PCR reaction was done at 94 °C, 60 °C, and 72 ° C, each for 20 sec, and 34 cycles. Relative mRNA levels were

computed with 2-delta CT, with β-actin as the internal reference. The HBV DNA was quantified with fluorescence quantitative PCR using SYBR Green supermax, with HBV expression plasma as quantitative standard, in a reaction system comprising 0.5 μL of 10 μM primer, 10 μL of SYBR Green supermax, 7 μL of double-distilled water and 2 μL of HBV DNA extract. The PCR reaction occurred at 94, 60, and 72 °C for 15, 30, and 20 sec, respectively, and in 39 cycles. A 10-fold dilution of HBV cccDNA sample in ddH_2O was used. The cccDNA level was determined using TaqMan probe-specific PCR. The PCR reaction system contained 0.5 μL of 10 μM primer, 0.5 μL of 10 μM probe, 10 μL GOTAQ ® Probe qPCR master mix, 6.5 μL of ddH_2O, and 2 μL of HBV cccDNA extract. The PCR reaction conditions were: 95 °C for 10 sec, 58 °C for 5 sec, 62 °C for 10 sec, 72 °C for 20 sec, and 44 cycles. The HBV expression plasma was used as quantitative standard. Taking the copy number log value of the standard sample as abscissa and CT value as ordinate, a standard curve was drawn, and the corresponding copy number of each sample was calculated from the standard curve.

Statistical analysis

GraphPad Prism 7.0 software was used for statistical analysis, and *t*-test of two independent samples was used to compare mean values between two groups. Single-factor ANOVA was used to compare means amongst multiple groups. All test results are presented as mean ± standard error of the mean (SEM) of a minimum of 3 replicates.

RESULTS

lncRNA pvt1 expression levels

The lncRNA pvt1 expressions in HBV-positive hepatoma cells were significantly higher than the corresponding expression levels in HBV-negative hepatoma cells, as shown in Figure 1.

HBV DNA contents of HepG2.2.15 cells after pvt1 silencing

In HepG2.2.15 cells, there were marked decreases in the contents of 3.2K HBV DNA after sipvt1 treatment, as shown in Figure 2.

Levels of HBsAg and HBeAg in HepG2.2.15 cells after pvt1 silencing

The expression levels of these factors were appreciably decreased by si-pvt1, as shown in results from ELISA assay (Table 1).

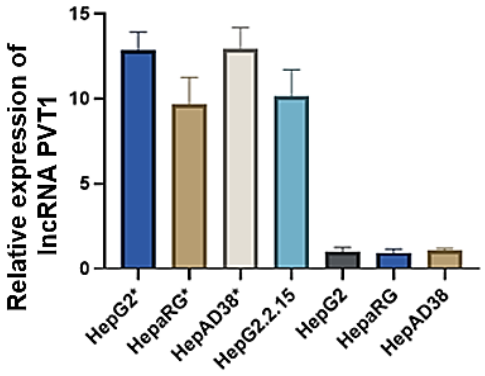

Figure 1: lncRNA pvt1 expression in HBV-positive and HBV-negative hepatocellular carcinoma (HCC) cells

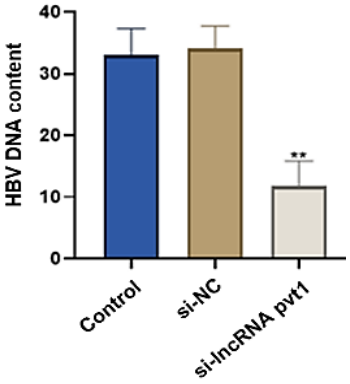

Figure 2: HBV DNA levels in HepG2.2.15 cells after pvt1 silencing. ***P* < 0.05, vs. si-NC cells

Table 1: HBsAg and HBeAg levels in HepG2.2.15 cells after pvt1 silencing

Group	HBsAg	HBeAg
Control	1.05±0.12	1.02±0.04
si-NC	1.06±0.07	1.07±0.06
Si-LNC pvt1	0.54±0.04**	0.47±0.03**

***P* < 0.05, vs. Si NC cells

Impact of si-pvt1 on STAT3 axis signal pathway

The STAT3 protein expression level was comparable in the three groups, but p-STAT3 protein expression was significantly decreased in si-lncpvt1 group, relative to those in si-NC and control groups. These data are shown in Table 2.

Table 2: Effect of 2pvt1 silencing on STAT3 axis signal pathway

Group	STAT3	P-STAT3
Control	1.06±0.08	0.58±0.06
si-NC	1.03±0.05	0.54±0.07
si-LNC pvt1	1.05±0.07	0.36±0.04**

***P* < 0.05, vs. si-NC cells

DISCUSSION

It has been reported that HBV infection advances to hepatic cirrhosis and HCC, and generally leads to poor prognosis in patients [6]. Thus, blocking of HBV replication is very important for arresting CHB-induced liver lesions. In patients with chronic HBV, antiviral therapy normalizes ALT levels in those with abnormal liver function, prevents disease progression, reduces associated complications, suppresses HBV viral load, and effectively blocks HBV transmission [8]. Therefore, it is of great significance to find biomarkers for early diagnosis and treatment of HBV infection. Long non-coding RNA (lncRNA) is associated with a variety of diseases, including HBV infection. Research has demonstrated that lncRNA regulates HBV replication. It regulates HBV transcription and replication by enhancing Sp1 transcription factors [7]. In particular, lncRNA pvt1 is associated with 1716 nuclei located in the chr8q24.21 region, and it is widely regarded as a cancer-promoting lncRNA [8].

Several investigations have revealed that lncRNA pvt1 is elevated in varieties of carcinomas *viz* bladder, liver, cervical, gastric, lung, and prostate cancers [9]. However, the role of lncRNA pvt1 in replication of HBV replication was hitherto unclear. Therefore, this investigation was done to unravel the influence of lncRNA pvt1 in the pathogenesis of HBV infection. Firstly, lncRNA pvt1 expressions in 4 HBV-positive hepatic carcinoma cells, and in corresponding HBV-negative liver cancer cell lines were determined using qRT-PCR.

Previous studies have shown that compared with HBV-negative hepatitis cells, lncRNA pvt1 expression in HBV-positive hepatitis cells is significantly up-regulated, suggesting that it is dysfunctional in various diseases [10]. In the present study, lncRNA pvt1 was significantly highly expressed in HBV-positive HCC, and it enhanced the migration and invasion of HBV-positive Hep3B cells, but it had no effect on HBV-negative HepG2 cells [11]. Therefore, it can be stated that the up-regulation of lncRNA pvt1 regulates HBV replication. Furthermore, to ascertain the influence of lncRNA pvt1 on HBV replication, small interference RNA (siRNA) was used to down-regulate its expression in HepG2.2.15 cells. It was found that the content of 3.2K HBV DNA was increased after si-pvt1 treatment. Moreover, HBV DNA HBeAg and HBsAg levels were significantly down-regulated in si-lncRNA pvt1 cells, relative to those in si-NC group, suggesting the involvement of lncRNA pvt1 in HBV replication process.

Previous studies have found that lncRNA pvt1 regulates the progression of a variety of cancers through a variety of signal pathways. For example, its silencing regulates miR-145 and impairs the survival, migratory potential, and apoptotic changes of esophageal cancer cells by the invasion of fscn1. Moreover, it has been reported that overexpressed miR-145 partially inhibited the growth, migration, and invasiveness of NSCLC cells by down-regulation of fscn1. It has been suggested that abnormal expression of miR-145 suppresses migratory and invasive potential of colorectal carcinoma cells. It is possible that miR-133b overexpression in esophageal squamous cell carcinoma cells decreases cancer cell survival and accelerates apoptosis. Interestingly, overexpression of miR-129 significantly suppressed the survival and invasive potential of esophageal squamous cell carcinoma [12].

It has been reported that the reduced expression of fscn1 decreased the viability and metastasis of gastric cancer cells. Indeed, lncRNA pvt1 plays a role as a tumor promoter, and a study has shown that it significantly reduced the survival and migratory and invasive potential while enhancing apoptosis of cervical carcinoma. Active lncRNA pvt1 promotes the invasiveness of esophageal carcinoma by enhancing epithelial-to-mesenchymal conversion [13]. In addition, down-regulation of lncRNA pvt1 inhibited the proliferation of renal carcinoma cells, and induced apoptosis. In a similar report, it has been revealed that when lncRNA casc9 was silenced, the migratory and invasive potential of EC cells *in vitro* was inhibited [14]. Thus, the down-regulation of lncRNA pvt1 induced high miR-145 expression levels which resulted in the inhibition of survival, migratory and invasive potential, thereby enhancing EC cell apoptosis by knocking down fscn1. However, the mechanism underlying lncRNA pvt1 regulation of replication of HBV replication was not hitherto clear.

At present, it has been found that lncRNA pvt1 activates the signal translation activator of transcription 3 (STAT3) signal, and it has been found that STAT3 occupies the lncRNA pvt1 promoter site, thereby activating its transcription. For example, studies have shown that lncRNA pvt1 enhanced angiogenesis by activating the STAT3/VEGFA axis [15]. A report has shown that lncRNA promoted hepatoblastoma by activating STAT3-induced cell cycle progression [16].

A large body of investigations has pointed out that STAT3 signals are crucial in HBV replication. For example, HBx activates STAT3 by inducing

IL-6 and activated STAT3 promotes HBV replication in synergy with hepatocyte nuclear factor 3 (HNF-3). The phosphorylation of STAT3 induces the formation of a hnf-3-p-stat3 isomerization complex which links with the homologous sequence in HBV enhancer 1, thereby promoting HBV replication. In addition, blocking STAT3 signal transmission through shRNAs enhances apoptosis of HBV-positive HCCs cells, mediates cell cycle stagnation, and inhibits HCC cell growth *in vitro* [17]. In this study, changes in the STAT3 axis signal pathway in HepG2.2.15 cells were studied after silencing lncRNA pvt1. It was found that the expression of STAT3 protein was comparable in the different groups, but p-STAT3 protein expression was significantly reduced in si-lncpvt1 group, relative to those in si-NC and control groups.

CONCLUSION

Silencing lncRNA pvt1 effectively inhibits STAT3 axis signal pathway, and thus inhibits HBV replication. The differential expressions of lncRNA pvt1 in HBV +ve and HBV-ve HEMA cells, and reduction in HBV DNA content in HepG2.2.15 cells after sipvt1 treatment are related to the STAT3 axis signal pathway. This finding may be beneficial for follow-up treatment of HBV.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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