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LncRNA H19/microRNA-675/PPARα axis regulates liver cell injury and energy metabolism remodelling induced by hepatitis B X protein via Akt/mTOR signalling

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ABSTRACT

Emerging evidence indicates that the lncRNAs/microRNA/mRNA axis plays important roles in a variety of diseases. This study was aimed to investigate the potential roles and underlying molecular mechanisms of lncRNA H19 and H19-derived miR-675 in regulating hepatitis B virus (HBV)-associated liver injury. mRNA and miR-675 levels were determined by quantitative real-time PCR (qRT-PCR), protein levels were determined by western blot, cell viability was measured by the MTT assay, cell apoptosis was measured by flow cytometry, inflammatory cytokine production was determined by ELISA, oxidative stress and energy metabolism were assessed by commercial kits, and the target relationship between PPARα and miR-675 was confirmed by the dual-luciferase reporter assay. The results showed that the expression of lncRNA H19 and miR-675 was up-regulated in patients with chronic hepatitis B (n = 20). Inhibition of lncRNA H19 or miR-675 in L02 cells increased cell viability, suppressed hepatitis B X protein (HBx)-induced cell apoptosis, inflammatory cytokine production, and oxidative stress, and remodelled energy metabolism. Furthermore, PPARα was found to be a target gene of miR-675. The expression of PPARα was down-regulated in patients with chronic hepatitis B, and there was a negative correlation between the expression of lncRNA H19 and PPARα, or between miR-675 and PPARα. Moreover, by knocking down the expression of PPARα, the actions (apoptosis, inflammatory factors, oxidative stress, and energy metabolism) of lncRNA H19 or miR-675 inhibition in HBx-induced L02 cells were at least partially reversed. In addition, HBx-induced elevated levels of p-Akt^{Ser473}, p-Akt^{Thr308} and p-mTOR^{Ser2448} were down-regulated by lncRNA H19 or miR-675 inhibition. Furthermore, PPARα knockdown partly reversed the down-regulated effects of H19 or miR-675 inhibition. Taken together, these data indicate that the lncRNA H19/miR-675/PPARα axis regulates liver cell injury and energy metabolism remodelling induced by HBx, which may be related to the modulation of Akt/mTOR signalling.

1. Introduction

Hepatitis B virus (HBV) is responsible for approximately 350 million chronic infections worldwide, and it is a leading cause of various liver diseases such as hepatitis, cirrhosis, hepatofibrosis, and hepatocellular carcinoma (HCC) (Ganem and Prince, 2004; Lavanchy, 2004; Oh and Park, 2015). The hepatitis B virus X protein (HBx), a small virally encoded protein, has been shown to be deeply related to HBV-associated pathogenesis (Feitelson and Duan, 1997). It has been implicated as a causative factor in the development of HCC by means of transactivating cellular oncogenes and promoting oncogenesis (Mingyue et al., 2015). However, in recent years, HBx has been reported to associate with affecting mitochondria physiology leading to hepatocyte death (Rahmani et al., 2000; Takada et al., 1999). Therefore,

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identifying its implication in the HBx-induced liver cell injury may help us better understand the pathogenesis, and provide potential new targets for chronic hepatitis B.

Increasing evidence has suggested that non-coding RNAs (ncRNAs) are functionally active in various physiological and pathological processes, which could be grouped into long non-coding RNAs (lncRNAs, longer than 200 nucleotides) and microRNAs (miRNAs, 18–22 nucleotides) according to the length. LncRNAs are transcripts that interact with multiple molecules, including DNA, RNA, and proteins (Zhang and Jeang, 2013). miRNAs regulate genes by binding to the 3' untranslated regions (UTRs) of target mRNAs, which leads to mRNA degradation or mRNA translation inhibition. Studies have indicated that both lncRNAs and miRNAs are involved in regulating HBV-associated liver diseases (Gong et al., 2016; Li et al., 2015; Xie et al., 2014; Yu et al., 2015).

The lncRNA H19 belongs to a highly conserved, imprinted gene cluster, and is deeply involved in embryonic development and growth control (Gabory et al., 2010). Increased lncRNA H19 expression has been observed in many inflammatory and organ fibrosis diseases, including ulcerative colitis, osteoarthritis, liver fibrosis, renal fibrosis, and pulmonary fibrosis (Yang et al., 2017). Moreover, it has been reported that H19 is a primary miRNA precursor for microRNA-675 (miR-675) (Cai and Cullen, 2007). The dysregulation of the H19/miR-675 axis has been found to be critically involved in several diseases, such as tumorigenesis (Tsang et al., 2010; Vennin et al., 2015), chronic obstructive pulmonary disease (Lewis et al., 2016), and diabetic cardiomyopathy (Li et al., 2016), suggesting that the function of H19 in some biological processes is mediated via miR-675. However, whether the H19/miR-675 axis participates in the regulation of HBV-associated hepatitis remains unknown.

In the present study, we aimed to determine the effects and underlying molecular mechanisms of the H19/miR-675 axis in HBx-induced liver cell injury in vitro, providing theoretical support to clarify the involvement of this axis in HBV-associated hepatitis. Our data indicate that the H19/miR-675 axis may be an effective therapeutic target for protecting the liver from HBV infection-induced injury.

2. Materials and methods

2.1. Patients

Patient liver samples were collected from 20 transcutaneous needle liver biopsy samples from patients with chronic hepatitis B. Normal tissue samples were collected from 20 patients who underwent surgery because of hepatic carcinoma. None of the patients included in the study were taking antiviral therapy or immunosuppressive drugs. All the patients were well informed. The study was approved by the ethics committee of Shandong Provincial Hospital affiliated with Shandong University.

2.2. Cell culture

Human liver cell line L02 cells and HEK293 cells were purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA). They were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C. L02 cells were transfected with a vector expressing the HBx, pcDNA3.1-HBx (containing a green fluorescent protein (GFP) protein, previously constructed in the laboratory) to induce liver injury.

2.3. Biochemical analyses

Levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using a fully automatic biochemical analyser (Roche Inc., USA) according to the manufacturer's protocols.

2.4. Cell transfection

Small interference RNA (siRNA) against lncRNA H19, PPARα, the miR-675 mimic, the miR-675 inhibitor, and the negative control were synthesised by GenePharma Co., Ltd. (Shanghai, China). The siRNA, miRNA mimic, or miRNA inhibitor was transfected into cells by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The sequences of H19 siRNA: 5′-CCCACAACAUGAAAGAA ACTT-3′ (sense) and 5′-AUUUCUUUCAUGU UGUGGGTT-3′ (antisense); the sequences of PPARα: 5′-GAGAUCGGCCUGGCCUUCUAAACAU-3′ (sense) and 5′-AUGUUUAGAAGGCCAGGCCGAUCUC-3′ (antisense).

2.5. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's guidelines. RNA was reverse transcribed into cDNA using a Prime-Script RT-PCR kit (TaKaRa, Dalian, China). The expression of H19 and PPARα was analysed by using SYBR Premix Ex Taq kit (Takara), and GAPDH was used as an endogenous control. The expression of miR-675 was analysed by using TaqMan microRNA assays (Applied Biosystems, Foster City, CA, USA), and U6 was used as an endogenous control. qRT-PCR was performed on an HT7900 Real-Time PCR System (Applied Biosystems). The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. Cell viability assay

The cell viability was detected by the MTT assay. Briefly, the cells were cultured in 96-well plates. 10 μl of MTT solution (Millipore, Billerica, MA, USA) was added to each well, followed by incubation for four hours. Then 150 μl of DMSO was added to dissolve for 15 min. The optical density was measured at 490 nm using a microplate reader (ThermoFisher Scientific, Waltham, MA, USA).

2.7. Western blot analysis

Protein homogenates were prepared from cells using RIPA buffer containing protease and phosphatase inhibitors. The protein concentration was quantified using the BCA protein assay kit (Pierce, USA). Then equal amounts of protein samples were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). After blocking by 5% non-fat milk at room temperature for one hour, the membranes were incubated with primary antibodies at 4 °C overnight: anti-cleaved-caspase-3, anti-Bax, anti-Bcl-2, anti-PPARα, anti-p-Akt^{Ser473}, anti-p-Akt^{Thr308}, anti-p-mTOR^{Ser2448}, anti-Akt, anti-mTOR, and anti-GAPDH, followed by incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature for one hour. The antibodies were purchased from Cell Signalling Technology (Beverly, MA, USA) or Abcam (Cambridge, MA, USA) and used at the manufacturers' recommended dilutions. The protein bands were visualised using an enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ, USA).

2.8. Apoptosis analysis

The cell apoptosis was determined by Annexin V-FITC/PI apoptosis detection kit (Jiancheng Biotech, Nanjing, China) according to the manufacturer's instructions. Briefly, the cells were washed in PBS and resuspended in binding buffer, and then labelled with Annexin V-FITC and PI at room temperature in the dark for 20 min. The apoptosis rates were analysed using a FACSCalibur (Becton Dickinson) within one hour.

2.9. Measurement of Caspase-3 activity

Caspase 3 activity was determined using the luminescent Caspase-

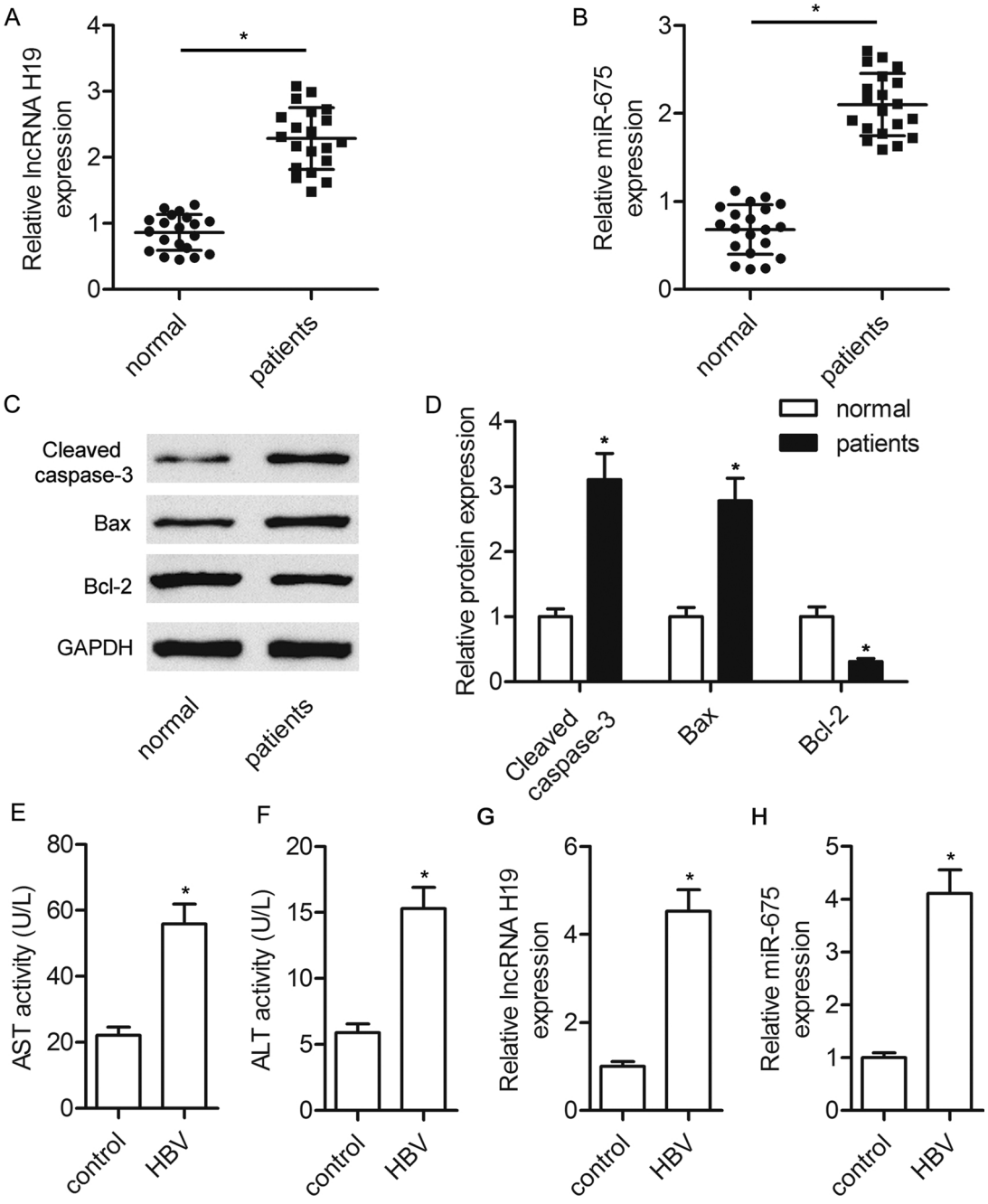

Fig. 1. LncRNA H19 and its-derived miR-675 was up-regulated in patients with chronic hepatitis B and in HBx-induced L02 cells. The expression of lncRNA H19 (A) and miR-675 (B) in 20 pairs of normal tissue and patient tissues with chronic hepatitis B was detected by qRT-PCR. (C) The representative images of cleaved caspase-3, Bax and Bcl-2 measured by Western blot in 4 pairs of normal tissues and patient tissues with chronic hepatitis B. (D) The relative expression of cleaved caspase-3, Bax and Bcl-2 were shown in histogram. Control group, normal cultured human liver cell line L02; HBV group, L02 cells transfected with pcDNA3.1-HBx. The levels of AST (E) and ALT (F) in cells were measured. The expression of lncRNA H19 (G) and miR-675 (H) in cells was detected by qRT-PCR (n = 4). *$P < 0.05$, compared with the normal or control group.

Glo 3 assay (Promega, Madison, WI, USA) according to the manufacturer's protocol.

2.10. Inflammatory cytokines detection

Concentrations of interferon-γ (IFN-γ), interleukin-6 (IL-6), and interleukin-18 (IL-18) were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

2.11. ROS measurement

Reactive oxygen species (ROS) levels were determined by 2,7-dichlorodihydrofluorescein diacetate probes (DCHF-DA, Beyotime, Shanghai, China). DCHF-DA oxidation produced the fluorescent 2,7-dichlorofluorescein (DCF). In brief, cells were incubated with 10 μM DCHF-DA for 20 min and then analysed by flow cytometry.

2.12. Measurement of SOD and MDA levels

The production of Malondialdehyde (MDA) and superoxide

Table 1
Characteristics of patients.

Group	CHB (patient liver samples)	HCC (para-carcinoma tissues-normal samples)
Individuals (n)	20	20
Gender (n)		
Male	13	15
Female	7	5
Age (yr)	46.6 ± 11.5	52.3 ± 8.4
HBV status (n)		
HBsAg+	20	4
HBsAg-	0	16

dismutase (SOD) were determined by commercial kits (Jiancheng Biotech) according to the manufacturer's guidelines.

2.13. Measurement of ATP concentration

The ATP concentration was quantified using rLuciferase/Luciferin reagent (Promega) in a luminometer according to the manufacturer's instructions. The ATP concentration was determined by plotting an ATP standard curve and was normalised to protein concentration.

2.14. Glucose consumption and lactate level

Glucose and lactate levels in the cell culture medium were determined using a glucose/lactate assay kit (Jiancheng Biotech) according to the manufacturer's instructions. Glucose consumption was calculated as the difference in the glucose concentration compared to the control (0 h). Glucose consumption and lactate levels were normalised against the cell numbers (Zeng et al., 2015).

2.15. Dual-luciferase reporter assay

The 3' UTR of PPARα containing the predicted miR-675 binding site was amplified by PCR (Forward primer: 5′-CTCGAGAGGUGGGAUGG AGACU-3′; Reverse primer: 5′-TTCGAACAGAGGAGACAGACCU-3′), and then cloned into the XhoI and HindIII sites of the pGL3 luciferase reporter vector, named as pGL3-PPARα 3' UTR vector. The mutant 3' UTR sequences without the miR-675 binding site were generated using a QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and used to generate the pGL3-PPARα 3' UTR mut vector. For the luciferase assay, HEK293 cells were plated into 96-well plates 24 h prior to transfection and then transfected with either a wild-type or mutant construct and the miR-675 mimic or negative control. After 48 h of transfection, luciferase activity was detected using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

2.16. Statistical analysis

Statistical analyses were carried out using the GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). All data are presented as mean ± SD of at least three independent experiments. Comparisons between the two groups were analysed by t-test. Comparisons among more than two groups were analysed by one-way analysis of variance (ANOVA) followed by the Bonferroni test. The negative correlation between lncRNA H19 and PPARα, or between miR-675 and PPARα in patient tissues was analyzed by line regression. $P < 0.05$ was considered statistically significant.

3. Results

3.1. LncRNA H19 and its-derived miR-675 was up-regulated in patients with chronic hepatitis B and HBx-induced L02 cells

As shown in Fig. 1A and Fig. 1B, compared with normal tissue, the expression of lncRNA H19 and its-derived miR-675 in the patient tissues with chronic hepatitis B were significantly up-regulated. Furthermore, western blot analyzes showed that compared with normal tissues, the expression of cleaved-caspase-3 and Bax was up-regulated, and the expression of Bcl-2 was down-regulated in the patient tissues with chronic hepatitis B (Fig. 1C and Fig. 1D). Then we transfected pcDNA3.1-HBx into L02 cells and measured the levels of AST and ALT. As shown in Fig. 1E and Fig. 1F, the levels of AST and ALT induced by the transfection of pcDNA3.1-HBx plasmids were significantly increased compared to the control cells, implying that HBx overexpression induced liver cell injury. Furthermore, compared with the control group, the expression of lncRNA H19 and miR-675 was found to be markedly up-regulated in the HBV group (Fig. 1G and Fig. 1H) (Table 1).

3.2. Inhibition of lncRNA H19 and miR-675 elevated cell viability and reduced HBx-induced cell apoptosis

To investigate the impact of lncRNA H19 and miR-675 on HBx-induced liver cell injury, we transfected H19 siRNAs or the miR-675 inhibitor into L02 cells, along with the pcDNA3.1-HBx, to suppress the HBx overexpression-induced up-regulation (Fig. 2A and Fig.2C). As H19 is a precursor of miR-675, compared with the HBV group, the transfection of H19 siRNAs also significantly reduced the expression of miR-675 (Fig. 2B). We further measured the cell viability and cell apoptosis to assess the impact of H19 and miR-675 on HBx-induced cell injury. As shown in Fig. 2D, compared with the control group, the cell viability in the HBV group was significantly decreased, which had a marked elevation by the transfection of H19 siRNAs or miR-675 inhibitors. Flow cytometry analyses showed that the inhibition of lncRNA H19 or miR-675 reduced the HBx overexpression-induced cell apoptosis rate (Fig. 2E). Western blot analyses further showed that compared with the control group, the expression of cleaved-caspase-3 and Bax was up-regulated, and the expression of Bcl-2 was down-regulated in the HBV group. Compared with the HBV group, the expression of cleaved-caspase-3 and Bax was down-regulated, and the expression of Bcl-2 was up-regulated in the H19 siRNA + HBV group or miR-675 inhibitor + HBV group (Fig. 2F). Moreover, the HBx overexpression-induced increased caspase-3 activity had an obvious reduction by the inhibition of lncRNA H19 or miR-675 (Fig. 2G).

3.3. Inhibition of lncRNA H19 and miR-675 decreased HBx-induced inflammatory factors and suppressed oxidative stress

We further measured the inflammatory factor production and oxidative stress level to assess the impact of lncRNA H19 and miR-675 on HBx-induced cell injury. As shown in Fig. 3A–3C, compared to the control group, the production of IFN-γ, IL-6 and IL-18 was significantly elevated by the HBx overexpression, and the elevation was obviously suppressed by the inhibition of lncRNA H19 or miR-675. As shown in Fig. 3D–3 F, compared to the control group, the production of ROS and MDA was increased, and the production of SOD was decreased in the HBV group. Compared with the HBV group, the production of ROS and MDA was decreased, and the production of SOD has increased in the H19 siRNA + HBV group or the miR-675 inhibitor + HBV group.

3.4. LncRNA H19 and miR-675 modulated HBx-induced energy metabolism remodelling

Furthermore, we measured the ATP concentration, glucose consumption, and lactate level to assess the remodelling of energy

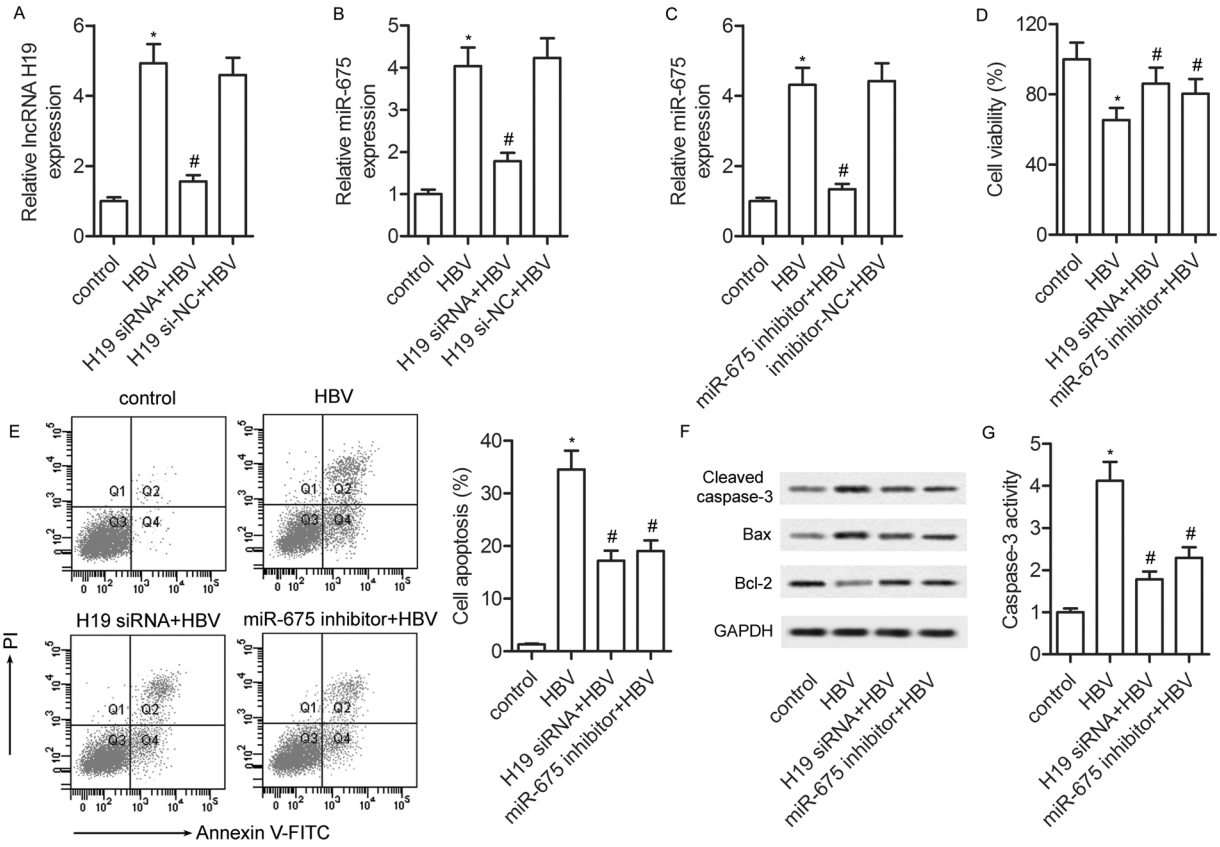

Fig. 2. Inhibition of lncRNA H19 and miR-675 elevated cell viability and reduced HBx-induced cell apoptosis. Control group, normal cultured L02 cells; HBV group, L02 cells transfected with pcDNA3.1-HBx; H19 siRNA (H19 si-NC) + HBV group, L02 cells co-transfected with H19 siRNAs (H19 siRNA negative control) and pcDNA3.1-HBx; miR-675 inhibitor (inhibitor-NC) + HBV group, L02 cells co-transfected with miR-675 inhibitors (inhibitor negative control) and pcDNA3.1-HBx. The expression of lncRNA H19 (A), and miR-675 (B and C) in cells was detected by qRT-PCR. (D) The cell viability was measured by the MTT assay. (E) The cell apoptosis was measured by flow cytometry. (F) The protein expression of cleaved caspase-3, Bax and Bcl-2 was measured by Western blot. (G) The caspase-3 activity was detected by a commercial kit (n = 4). $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the HBV group.

metabolism. As shown in Fig. 4A–4C, compared to the control group, the overexpression of HBx induced a reduction in the ATP concentration, but increased glucose consumption and elevated the lactate levels. Compared with the HBx group, the inhibition of H19 or miR-675 significantly increased the ATP concentration and decreased glucose consumption and the lactate levels.

3.5. PPARα is a target gene of miR-675 and is down-regulated in patients with chronic hepatitis B

In Fig. 5A, the potential targeting sequences between miR-675 and PPARα are shown. The dual-luciferase reporter assay showed that compared to the mimic-NC, the miR-675 mimic transfection markedly decreased the luciferase activity of the plasmid containing the 3' UTR of PPARα, but had no effect on the luciferase activity of the plasmid containing the mutant 3' UTR of PPARα. As shown in Fig. 5B and Fig. 5C, the mRNA and protein expression of PPARα was down-regulated in L02 cells transfected with miR-675 mimic, which was up-regulated in L02 cells transfected with the miR-675 inhibitor. We then found that compared to normal tissues, the expression of PPARα in the patient tissues with chronic hepatitis B were significantly down-regulated (Fig. 5D). Moreover, there was a negative correlation between the expression of lncRNA H19 and PPARα, or between miR-675 and PPARα in the patient tissues (Fig. 5E and Fig. 5F).

3.6. PPARα knockdown partly reverses the effects of lncRNA H19 or miR-675 inhibition

To investigate whether the effects of lncRNA H19 or miR-675 inhibition on HBV-induced cell injury were associated with PPARα, we knocked down the PPARα expression by transfecting PPARα siRNAs into L02 cells under the condition of H19 or miR-675 inhibition (Fig. 6A). Then we again measured the cell viability, cell apoptosis, inflammatory cytokine production, ROS level, and energy metabolism; As shown in Fig. 6B–6H, PPARα knockdown partly reversed the effects of H19 or miR-675 inhibition on the above cell functions. Compared with the H19 siRNAs + HBV group, the decreased cell viability, elevated caspase-3 activity, increased production of IFN-γ, IL-6, and ROS, reduced ATP concentration and increased glucose consumption were found in H19 siRNA + PPARα siRNA + HBV group. Consistent with that, compared with the miR-675 inhibitor + HBV group, the same results were found in the miR-675 inhibitor + PPARα siRNA + HBV group. Moreover, compared with the HBV group, the decreased caspase-3 activity, decreased production of IFN-γ, IL-6, and ROS, increased ATP concentration and reduced glucose consumption were found in the PPARα + HBV group or the H19 siRNA + miR-675 inhibitor + PPARα + HBV group (Supplementary Fig. 4).

3.7. lncRNA H19/miR-675/PPARα axis regulates Akt/mTOR signalling

To investigate the underlying signalling pathway mediated the effects of lncRNA H19/miR-675/ PPARα axis, we measured the protein levels of Akt/mTOR signalling. As shown in Fig. 7A–7D, compared with

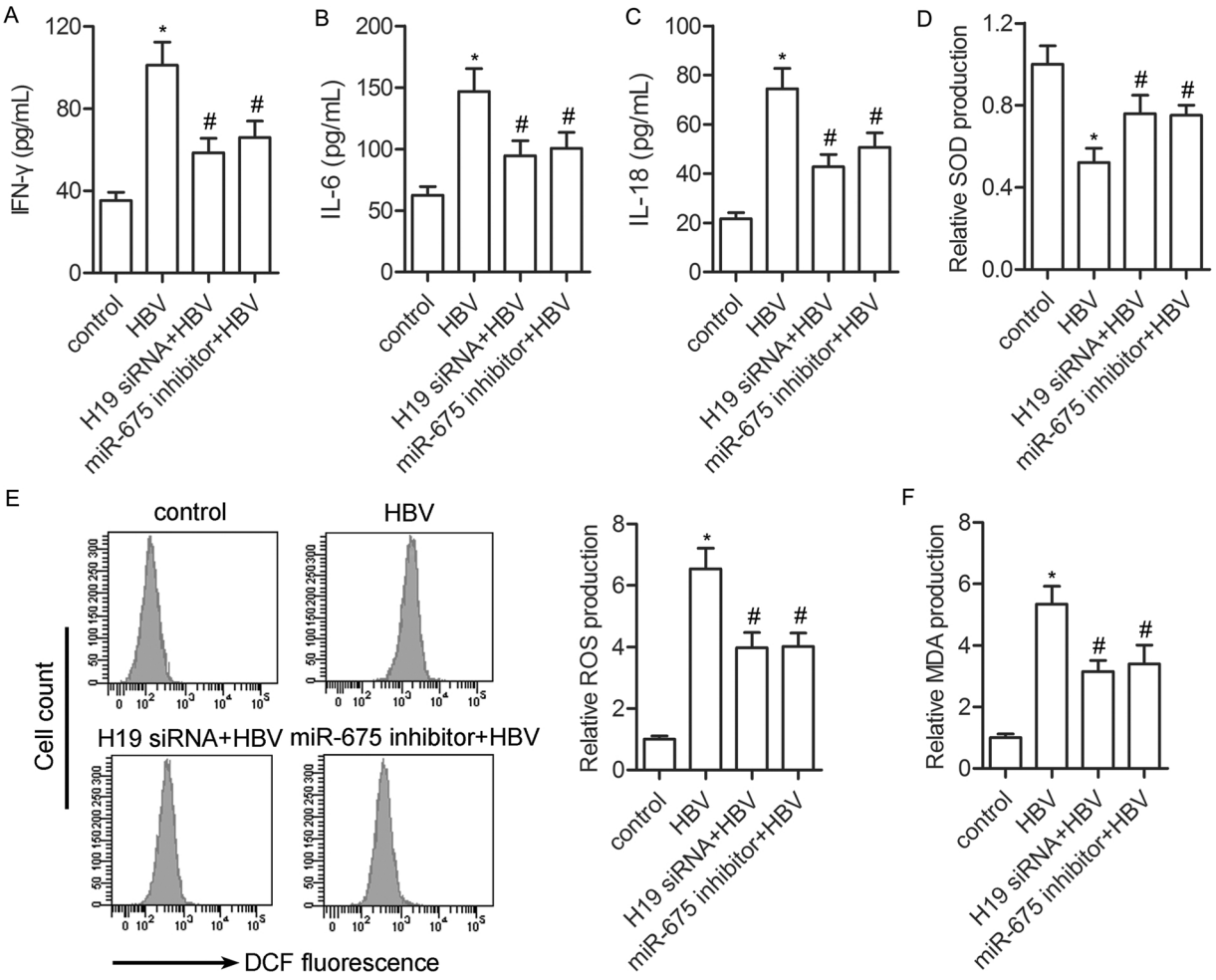

Fig. 3. Inhibition of lncRNA H19 and miR-675 decreased HBx-induced inflammatory factors and suppressed oxidative stress. Control group, normal cultured L02 cells; HBV group, L02 cells transfected with pcDNA3.1-HBx; H19 siRNA + HBV group, L02 cells co-transfected with H19 siRNAs and pcDNA3.1-HBx; miR-675 inhibitor + HBV group, L02 cells co-transfected with miR-675 inhibitors and pcDNA3.1-HBx. The levels of IFN-γ (A), IL-6 (B) and IL-18 (C) were detected by ELISA commercial kits. (D) The ROS production was measured by flow cytometry using DCHF-DA probes. The production of MDA (E) and SOD (F) was measured by commercial kits (n = 4). $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the HBV group.

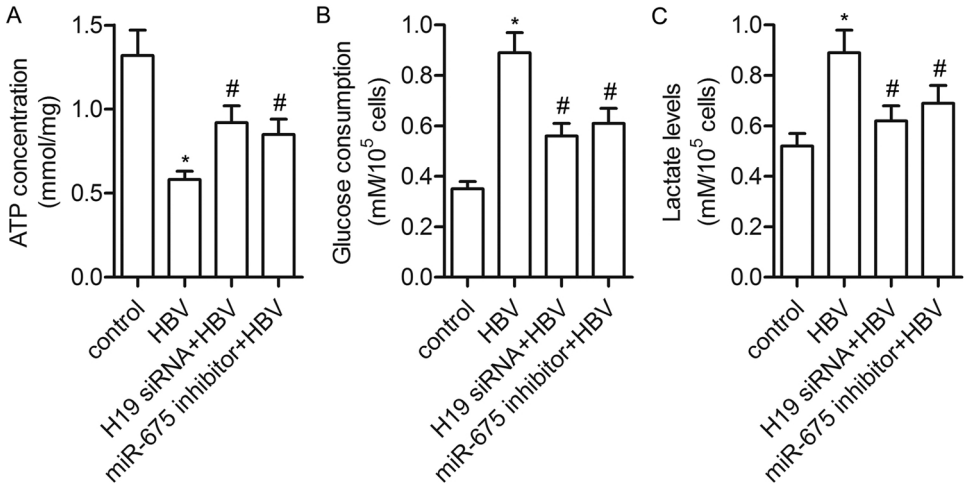

Fig. 4. LncRNA H19 and miR-675 modulated HBx-induced energy metabolism remodelling. Control group, normal cultured L02 cells; HBV group, L02 cells transfected with pcDNA3.1-HBx; H19 siRNA + HBV group, L02 cells co-transfected with H19 siRNAs and pcDNA3.1-HBx; miR-675 inhibitor + HBV group, L02 cells co-transfected with miR-675 inhibitors and pcDNA3.1-HBx. (A) The ATP concentration was quantified using rLuciferase/Luciferin reagent. The glucose consumption (B) and lactate level (C) was determined by a commercial kit (n = 4). $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the HBV group.

the control group, HBx overexpression induced elevated levels of p-Akt^{Ser473}, p-Akt^{Thr308}, and p-mTOR^{Ser2448}, which were down-regulated by lncRNA H19 or miR-675 inhibition. Furthermore, PPARα knock-down partly reversed the down-regulated effects of lncRNA H19 or miR-675 inhibition. Compared with the H19 siRNA + HBV group or miR-675 inhibitor + HBV group, the levels of p-Akt^{Ser473}, p-Akt^{Thr308}, and p-mTOR^{Ser2448} were up-regulated in the H19 siRNA + PPARα siRNA + HBV group or miR-675 inhibitor + PPARα siRNA + HBV group. The protein expression of Akt and mTOR showed no significant changes in the above experiments. Furthermore, compared with the

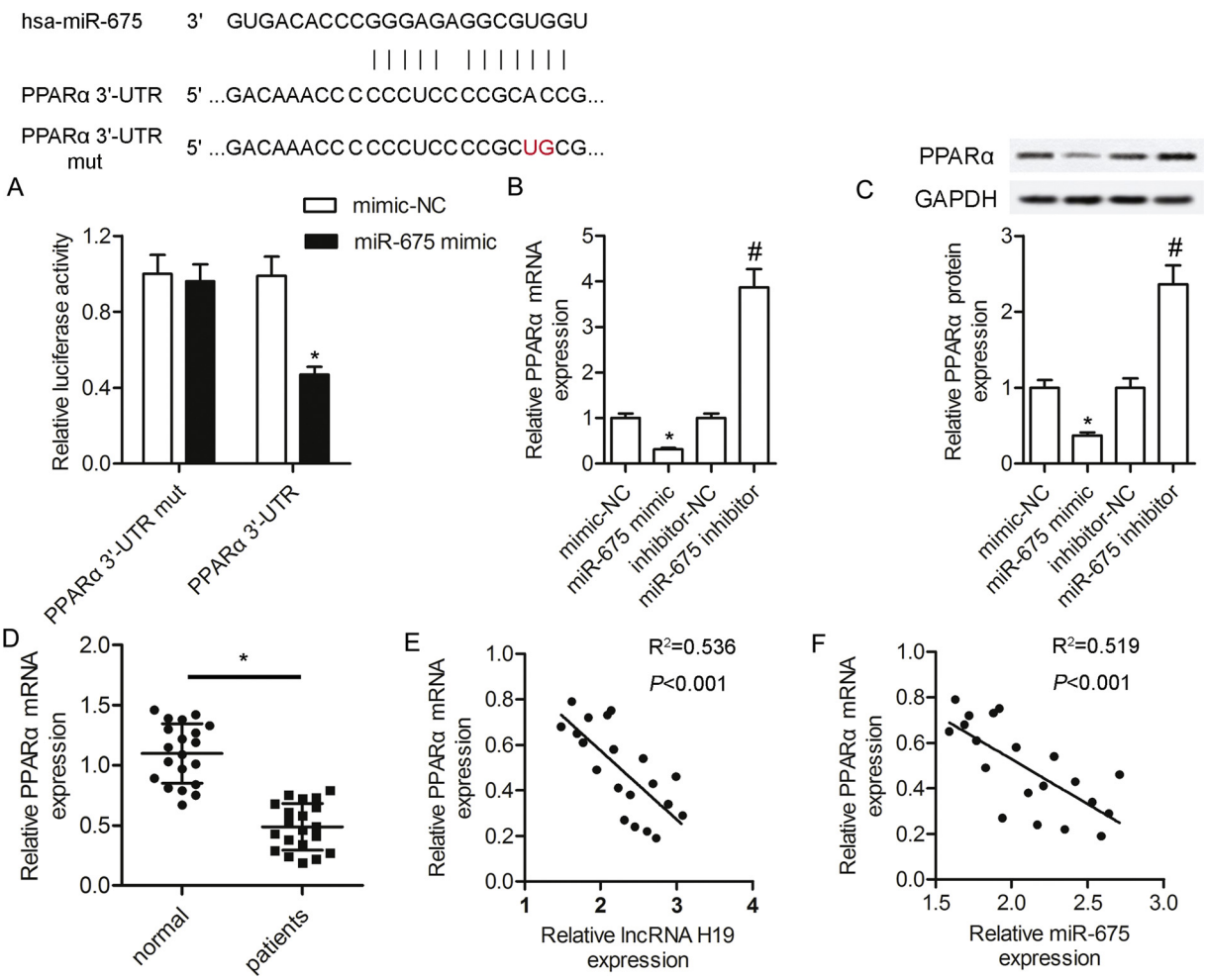

Fig. 5. PPARα was a target gene of miR-675 and was down-regulated in patients with chronic hepatitis B. (A) Predicted 3' UTR sequence of PPARα containing miR-675 binding sites. (B) The miR-675 mimic or mimic-NC were co-transfected into HEK293 T cells with the reporter plasmids that containing PPARα 3' UTR or PPARα 3' UTR mut, along with a control Renilla luciferase pRL-TK vector. Luciferase activity was analysed 48 h after transfection and normalised to Renilla luciferase activity (n = 4). $^{*}P < 0.05$, compared with the mimic-NC group. The (B) mRNA and (C) protein expression of PPARα in L02 cells transfected with miR-675 mimic or inhibitor was determined by qRT-PCR and Western blot, respectively (n = 3). $^{*}P < 0.05$, compared with the mimic-NC group; $^{\#}P < 0.05$, compared with the inhibitor-NC group. (D) The expression of PPARα in 20 pairs of normal tissues and patient tissues with chronic hepatitis B was detected by qRT-PCR. The negative correlation between lncRNA H19 and PPARα (E), or between miR-675 and PPARα (F) in 20 patient tissues was shown.

control group, the levels of p-Akt^{Ser473}, p-Akt^{Thr308}, and p-mTOR^{Ser2448} were down-regulated in the H19 siRNA + miR-675 inhibitor + PPARα group (Supplementary Fig. 3) (Fig. 8).

4. Discussion

As a multifunctional trans-activator protein, HBx plays a crucial role in HBV-associated liver diseases. However, the precise mechanisms by which HBx regulates liver cell injury remain unclear. Given the implication of H19 in many inflammatory disorders, we speculated whether it was involved in HBV-associated hepatitis and liver cell injury.

In the present study, we first found that compared with normal tissue, lncRNA H19 was significantly up-regulated in the patients' tissues with chronic hepatitis B, suggesting that the up-regulation of lncRNA H19 was associated with the progression of chronic hepatitis B. As lncRNA H19-derived miR-675, its expression was also up-regulated in patient tissues with chronic hepatitis B, suggesting that miR-675 may mediate the effects of lncRNA H19. We established a liver injury cell model by transfecting pcDNA3.1-HBx plasmids into L02 cells. The increased levels of AST and ALT were found following HBx overexpression, suggesting that HBx overexpression induced liver cell injury, which was consistent with the previous study (Liang et al., 2007). Furthermore, the expression of lncRNA H19 and miR-675 was markedly up-regulated by HBx overexpression. These results demonstrated that the lncRNA H19 and miR-675 axis was involved in HBV-associated hepatitis and liver cell injury.

Hepatic cell death is an important cause of fatality for patients with HBV infection (Liang et al., 2007). It has been reported that HBx could induce cell death and sensitise cells to apoptosis (Hong-Ying et al., 2005; Oh and Park, 2015; Terradillos et al., 2002). Kim et al. reported that HBx could interact with c-FLIP to abrogate its apoptosis-inhibitory function, and thus, render the cell hypersensitive towards the TNF-a apoptotic signal even below threshold concentrations (Kim and Seong, 2003). Shirakata et al. showed that HBx could cause a loss of mitochondrial membrane potential and subsequently induce mitochondrial-dependent cell death (Shirakata and Koike, 2003). In the present study, the apoptosis markers (cleaved-caspase-3 and Bax) were up-regulated, and the expression of Bcl-2 was down-regulated in the patient tissues with chronic hepatitis B. Furthermore, the cell viability was significantly decreased, and the cell apoptosis was significantly elevated by the HBx overexpression in L02 cells, which was consistent with one previous study (Hong-Ying et al., 2005). To investigate the implication of lncRNA H19 and miR-675 in the process, we inhibited their expression by transfecting H19 siRNAs or miR-675 inhibitors,

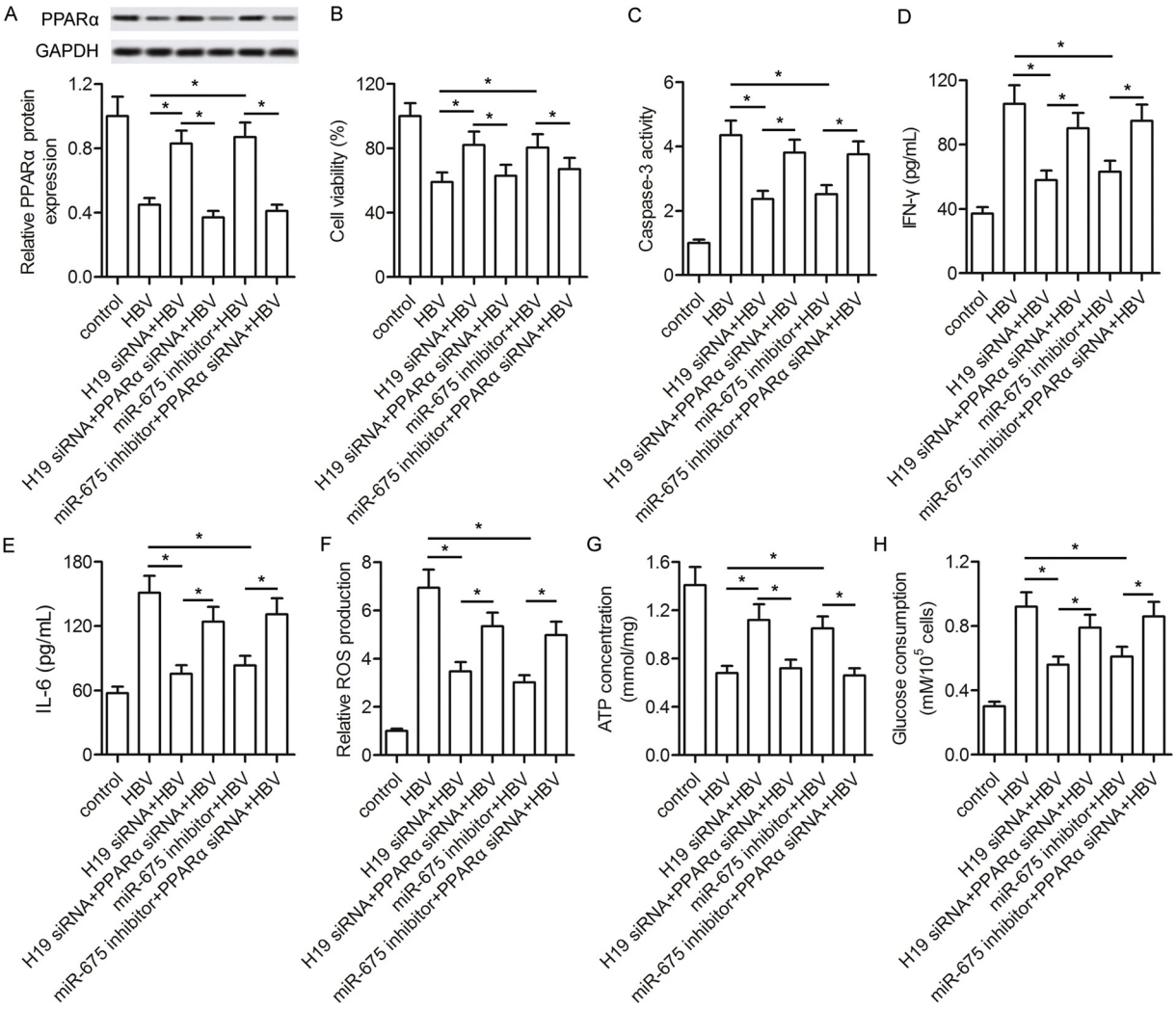

Fig. 6. PPARα knockdown partly reversed the effects of lncRNA H19 or miR-675 inhibition. Control group, normal cultured L02 cells; HBV group, L02 cells transfected with pcDNA3.1-HBx; H19 siRNA + HBV group, L02 cells co-transfected with H19 siRNAs and pcDNA3.1-HBx; H19 siRNA + PPARα siRNA + HBV group, L02 cells co-transfected with H19 siRNAs, PPARα siRNAs and pcDNA3.1-HBx; miR-675 inhibitor + HBV group, L02 cells co-transfected with miR-675 inhibitors and pcDNA3.1-HBx; miR-675 inhibitor + PPARα siRNA + HBV group, L02 cells co-transfected with miR-675 inhibitors, PPARα siRNAs and pcDNA3.1-HBx. (A) The protein expression of PPARα was determined by Western blot. (B) The cell viability was measured by MTT assay. (C) The caspase-3 activity was detected by a commercial kit. The levels of IFN-γ (D) and IL-6 (E) were detected by ELISA commercial kits. (F) The ROS production was measured by flow cytometry using DCHF-DA probes. (G) The ATP concentration was quantified using rLuciferase/Luciferin reagent. (H) The glucose consumption was determined by a commercial kit (n = 3). $^{*}P < 0.05$.

respectively. Inhibition of lncRNA H19 or miR-675 elevated the cell viability and reduced HBx overexpression-induced cell apoptosis. During mitochondrial apoptosis, the increased cleaved caspase-3 has been shown to contribute to the cleavage of cellular target proteins (Riedl and Shi, 2004). It is also well known that the Bcl-2 family of proteins plays a crucial role in apoptosis. Bcl-2 blocks apoptosis by preserving the integrity of the outer mitochondrial membrane, directly or indirectly, thus preventing the release of cytochrome c. Bcl-2 has been reported to prevent hepatocyte apoptosis (De et al., 1999; Lacronique et al., 1996). The pro-apoptotic member Bax induces apoptosis by causing cytochrome c release. Liang et al. indicated that Bax is a critical regulator of HBx-associated hepatocyte death (Liang et al., 2007). In the present study, we further found that HBx overexpression up-regulated the expression of cleaved-caspase-3 and Bax, and down-regulated the expression of Bcl-2, which was reversed by the inhibition of lncRNA H19 or miR-675. These results suggest that lncRNA H19/miR-675 axis regulated HBx-induced cell apoptosis in a mitochondria-dependent manner.

HBV is largely non-cytopathic, and liver injury might be mediated by HBV-induced immune responses (Chang and Lewin, 1992; Chisari and Ferrari, 1995). Experimental evidence suggests that immune response factors, especially the pro-inflammatory cytokines, play an important role in liver injury induced by HBV (Falasca et al., 2006). IL-6 and IL-18 are identified as pro-inflammatory cytokines that are involved in viral clearance, and in metabolic and viral hepatic diseases, respectively (Li et al., 2004; Vecchiet et al., 2005). In the present study, we found that the production of IFN-γ, IL-6, and IL-18 was significantly elevated by the HBx overexpression, and the elevation was obviously suppressed by the inhibition of lncRNA H19 or miR-675. HBx expression has been shown to induce oxidative stress via calcium signalling and cellular kinases (Bouchard et al., 2001; Waris et al., 2001). Moreover, the induction of apoptosis has been suggested to be closely associated with the generation of ROS (Srisuttee et al., 2011). In the present study, inhibition of lncRNA H19 or miR-675 was found to suppress HBx-induced oxidative stress by decreasing ROS and MDA levels and increasing SOD production. Taken together, these results suggest that lncRNA H19/miR-675 axis regulated HBx-induced inflammatory factors and oxidative stress. In addition, energy metabolism

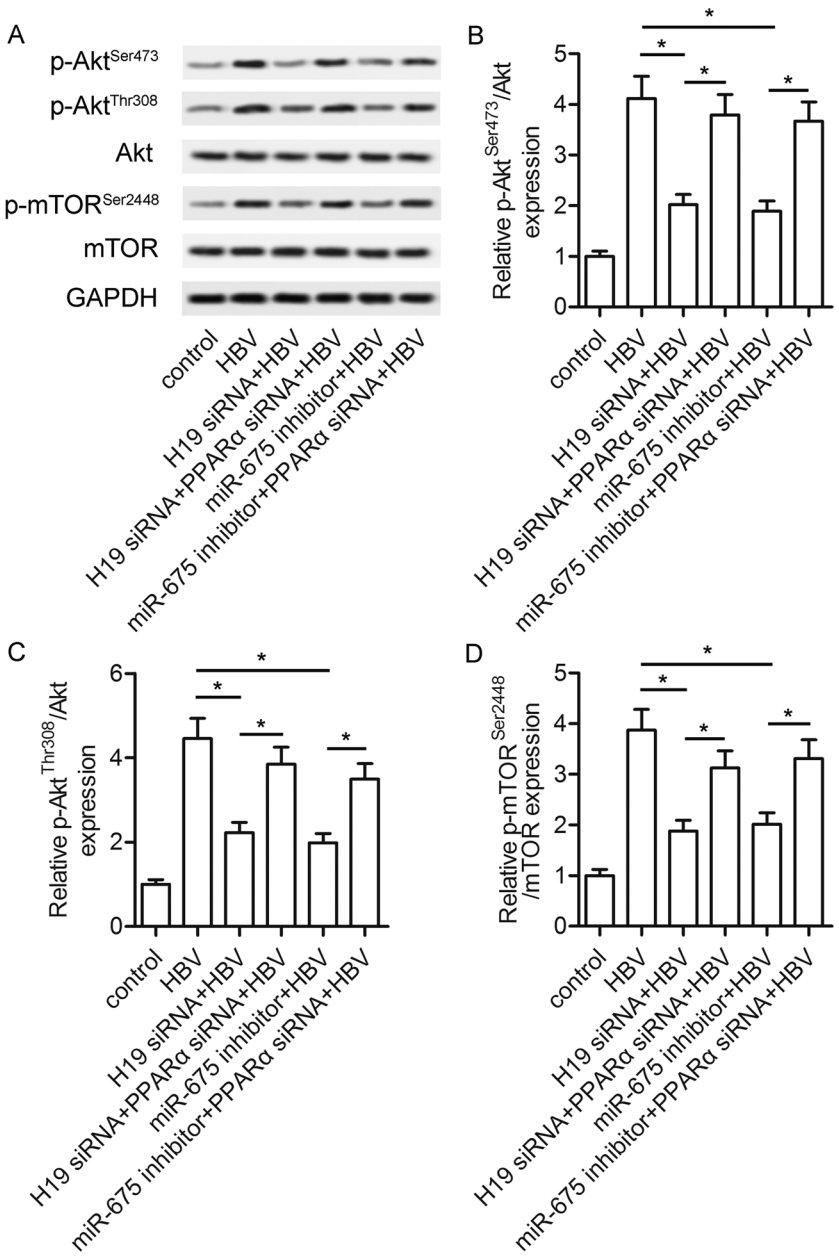

Fig. 7. LncRNA H19/miR-675/ PPARα axis regulated Akt/mTOR signalling. Control group, normal cultured L02 cells; HBV group, L02 cells transfected with pcDNA3.1-HBx; H19 siRNA + HBV group, L02 cells co-transfected with H19 siRNAs and pcDNA3.1-HBx; H19 siRNA + PPARα siRNA + HBV group, L02 cells co-transfected with H19 siRNAs, PPARα siRNAs and pcDNA3.1-HBx; miR-675 inhibitor + HBV group, L02 cells co-transfected with miR-675 inhibitors and pcDNA3.1-HBx; miR-675 inhibitor + PPARα siRNA + HBV group, L02 cells co-transfected with miR-675 inhibitors, PPARα siRNAs and pcDNA3.1-HBx. (A) The protein levels of p-Akt^{Ser473}, p-Akt^{Thr308}, Akt, p-mTOR^{Ser2448}, and mTOR were determined by Western blot. The relative expression of p-Akt^{Ser473} (B), p-Akt^{Thr308} (C), and p-mTOR^{Ser2448} (D) were shown in histograms (n = 3). *$P < 0.05$.

plays a crucial role in sustaining normal cellular functions in all cellular biochemical reactions (Zeng et al., 2015). Abnormal energy metabolism has been revealed to be closely associated with chronic hepatitis B (Fan et al., 2006; Zhao et al., 2015). Our results showed that lncRNA H19/miR-675 axis regulated HBx-induced remodelling of energy metabolism, at least partly by modulating the ATP concentration, glucose consumption, and lactate level.

To investigate the underlying molecular mechanisms of the H19-miR-675 axis in HBx-induced cell injury, we predicted the putative target genes of miR-675 using Miranda and TargetScan. PPARα was chosen for our focus, which has been reported to be involved in HBV-associated liver diseases (Choi et al., 2005; Xiao-Tao et al., 2011). The subsequent dual-luciferase reporter assay confirmed that miR-675 directly targets the 3'-UTR of the PPARα mRNA. We then found that the expression of PPARα in the patient tissues with chronic hepatitis B was significantly down-regulated, and there was a negative correlation between the expression of lncRNA H19 and PPARα, or between miR-675 and PPARα. These results suggest that H19/miR-675/PPARα axis may play a crucial role in chronic hepatitis B. It has been reported that PPARs belong to the nuclear receptor superfamily of ligand-activated transcription factors, which regulate lipid/lipoprotein metabolism, glucose homeostasis, amino acid metabolism, and inflammation (Desvergne and Wahli, 1999; Francis et al., 2003). Emerging investigations have shown that PPARs are closely associated with the regulation of immunity, inflammatory reactions, and energy metabolism (Alleva et al., 2002; Chen and Yang, 2014; Delerive et al., 2001; Rosen, 2003). PPARα has also been proved to play a role in the expression or activation of antioxidant enzymes (Toyama et al., 2004). Therefore, we speculated that PPARα mediated the biological functions of H19-miR-675 axis on HBx-induced cell injury. To confirm to speculation, we knocked down the PPARα expression under the condition of H19 or miR-675 inhibition. We again measured the cell viability, cell apoptosis, inflammatory cytokine production, ROS level, and energy metabolism, and found that PPARα knockdown partly reversed the effects of H19 or miR-675 inhibition on those cellular functions. Taken together, these data indicate that PPARα, at least, partially mediate the effects of the H19/miR-675 axis in the HBx-induced liver cells.

The mTOR is a serine/threonine kinase member of the PI3K/Akt

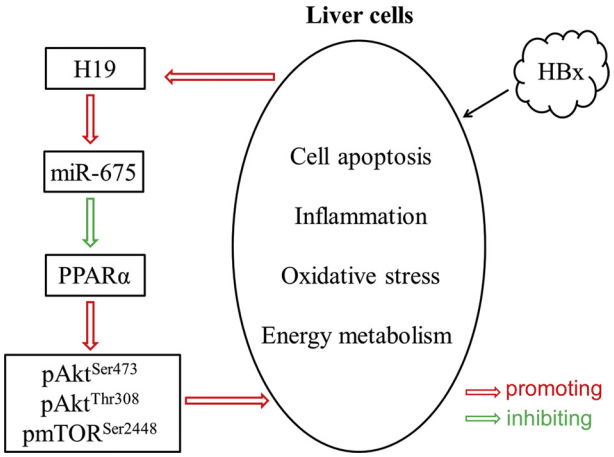

Fig. 8. A schematic diagram outlining the mode of action of H19/miR-675/PPARa axis in HBx-induced liver cells. HBx overexpression up-regulates the expression of lncRNA H19 and miR-675, and thus suppresses PPARα expression, which further activates the Akt/mTOR signaling pathway. And H19/miR-675/PPARa/Akt/mTOR axis collectively promotes HBx-induced liver cell injury, such as cell apoptosis, inflammation, oxidative stress and energy metabolism remodeling.

signalling pathway and is phosphorylated by activated Akt (Hassan et al., 2013), which acts as an important regulator of eukaryotic cell growth and is involved in regulating a variety of cellular functions, including proliferation, apoptosis, and differentiation (Betz and Hall, 2013). Furthermore, mTOR is identified as an essential sensor and controller of cellular energy metabolism (Polak and Hall, 2009; Tokunaga et al., 2004). The aberrant activation of the mTOR pathway by HBx has been discovered in chronic hepatitis B (Wang et al., 2015). We, thus investigated whether the activity of H19/miR-675/PPARα axis in HBx-induced cell injury was associated with the modulation of Akt/mTOR signalling. In the present study, we found that lncRNA H19 or miR-675 inhibition down-regulated HBx overexpression-induced elevated levels of p-Akt^{Ser473}, p-Akt^{Thr308}, and p-mTOR^{Ser2448}. Moreover, PPARα knockdown partly reversed the down-regulated effects of H19 or miR-675 inhibition. These results suggest that the activity of H19/miR-675/PPARα axis in HBx-induced cell injury was associated with the modulation of Akt/mTOR signalling.

In summary, these results demonstrate that the abnormal expression of lncRNA H19, miR-675, and PPARα in the patient tissue with chronic hepatitis B. Inhibition of lncRNA H19 or miR-675 in vitro elevated cell viability, suppressed HBx-induced cell apoptosis, inflammatory factor production, and oxidative stress, and remodelled energy metabolism via regulating PPARα expression, which may be closely associated with Akt/mTOR signalling. Therefore, targeting H19/miR-675/PPARα axis may provide new insights into understanding the molecular mechanisms of chronic hepatitis B, and reveal a potential therapeutic strategy for its treatment.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.09.006.

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