Short hairpin RNA attenuates liver fibrosis by regulating the peroxisome proliferator-activated receptor- γ and nuclear factor- κ B pathways in hepatitis B virus-induced liver fibrosis in mice

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Abstract

Background: Progressive liver fibrosis, caused by chronic viral infection and metabolic disorders, results in the development of cirrhosis and hepatocellular carcinoma. However, no antifibrotic therapies have been approved to date. In our previous study, adeno-associated virus (AAV) short hairpin RNAs (shRNAs) targeting hepatitis B virus (HBV) and transforming growth factor (TGF)- β administration could persistently inhibit HBV replication and concomitantly prevent liver fibrosis. However, the differentially expressed proteins and critical regulatory networks of AAVshRNA treatment remain unclear. Accordingly, in this study, our major goal was we aimed to analyze differentially expressed proteins in the liver of AAV-shRNAs-treated mice with HBV infection and liver fibrosis using isobaric tags for relative and absolute quantitation (iTRAQ)-based quantitative proteomics and to elucidate the underlying antifibrotic mechanisms. **Results:** In total 2743 proteins were recognized by iTRAQ-based quantitative proteomics analysis. Gene ontology analysis suggested that the differentially expressed proteins were mostly participated in peptide metabolism in the biological process category, cytosolic ribosomes in the cell component category, and structural constituents of ribosomes in the molecular function category. Kyoto Encyclopedia of Genes and Genomes pathway analysis indicated that oxidative stress and the peroxisome proliferator-activated receptor (PPAR) signaling pathway were activated after treatment. Verification studies showed that AAVshRNAs inhibited hepatic stellate cell activation and inflammation by suppressing nuclear factor- κ B p65 phosphorylation and α -smooth muscle actin expression via upregulation of PPAR- γ . Hepatocytes steatosis was also decreased by activating PPAR signaling pathway and improving lipid metabolism. TGF- β level was decreased owing to increase PPAR- γ expression and directly inhibition using AAVshRNAs targeting TGF- β . TGF- β -induced oxidative stress was suppressed by increasing glutathione S-transferase Pi 1 and reducing peroxiredoxin 1. **Conclusions:** Our results indicated that AAV-shRNAs were effective for modulating liver fibrosis by reducing oxidative stress, inflammation and activating PPAR signaling pathway.

Background

Hepatitis B virus (HBV) infection is a major health problem, which cause acute and chronic hepatitis, and progress to cirrhosis, and hepatocellular carcinoma (HCC) [1-4]. More than 780,000 people die from HBV infection or related complications each year[5]. Approximately 25-40% of cases of liver fibrosis result in cirrhosis or HCC [6, 7]. Moreover 70-90% of clinical HCC cases are related to advance liver fibrosis or cirrhosis[8].

Liver fibrosis is a common wound healing process response to chronic liver injury via excessive production and deposition of extracellular matrix (ECM)[9]. Hepatic stellate cells (HSCs) are major producers of matrix components and play pivotal roles in regulating the production and secretion of the ECM [10]. Typically, HSCs remain in a quiescent state and function in the storage of vitamin A. Upon liver injury, HSCs may undergo transdifferentiation, transform into highly proliferative myofibroblast-like cells, and acquire fibrogenic properties, including expression of α -smooth muscle actin (α -SMA), type I collagen, and type III collagen, which are vital components of the ECM[11]. Although inhibiting of HSC activation

has been proposed as therapeutic strategy in anti-fibrosis treatment of fibrosis[12], novel approaches for uncovering the mechanisms of liver fibrosis and for development of antifibrotic treatments are still challenging.

Transforming growth factor(TGF)- β is a critical mediator that plays important roles in human fibrogenesis [13]. Many studies have shown that TGF- β signaling through the Smad pathway and reactive oxygen (ROS) imbalance are responsible for liver fibrosis [14-17]. TGF- β has also been shown to inhibit the antioxidant system and hence induce oxidative stress or redox imbalance [18-20]. Such a redox imbalance dedicates importantly to TGF- β related pathophysiologic effects containing fibrosis[16]. Therapeutics targeting TGF- β -induced ROS-dependent cellular signaling may be a new therapeutic method in the treatment of fibrotic disorders. However, the mechanisms underlying of liver fibrosis associated with redox-sensitive targets remain unclear.

Peroxisome proliferator-activated receptors (PPARs) including PPAR- α , PPAR- β/δ , and PPAR- γ , are ligand-activated transcription factors belonging to the nuclear hormone receptor family [21-25]. Previous studies have shown that PPAR- γ is predominantly present in adipose tissue, and plays an important role in many biological processes, such as adipogenesis, cell differentiation, cell growth regulation and inflammatory reactions[26, 27]. PPAR- γ has also been shown to be a driver of liver fibrosis in hepatic tissues, and its activation promotes insulin sensitivity and inhibits the transformation of HSCs from a quiescent to activated state [28-31]. Previous studies have indicated that the activation of PPAR- γ can reduce connective tissue growth factor expression induced by TGF- β 1 in HSCs [32] and that the PPAR- γ agonist rosiglitazone can enhance PPAR- γ expression in activated HSCs, leading to reduced oxidative stress and decreased expression of α -SMA and collagen I[33].

The transcriptional regulator nuclear factor (NF)- κ B is an important mediator of inflammatory signals in response to stimulation [34-36]. Many reports have shown that upregulation of NF- κ B stimulates HSC proliferation and inhibits HSC apoptosis, playing a key role in fibrogenesis [36-38]. Moreover, NF- κ B can induce the expression of inflammatory factors (TGF- β , interleukin [IL]-6, and tumor necrosis factor- α), which play pivotal role in the development of liver fibrosis [36, 37, 39, 40]. Excessive production of ROS also can induce phosphorylation of NF- κ B, which then migrates to the nucleus to increase the transcription of pro-inflammatory cytokines and results in HSC activation [41]. Accordingly, reducing the activation of NF- κ B can lead to inhibition of HSC activation and ECM production [42, 43].

Recent studies have uncovered many mechanisms that mediate liver fibrosis. However, no highly effective antifibrotic therapies are currently available. In our previous study, we found that adeno-associated virus (AAV) short hairpin RNAs (shRNAs) targeting HBV and TGF- β inhibited HBV replication and liver fibrosis in an HBV-induced liver fibrosis mouse model[44]. Removal of the causative agent (HBV) by RNA interference (RNAi) is an effective strategy for treating HBV-induced liver fibrosis, whereas inhibition of the TGF- β pathway alone is not effective. Our study showed the advantages of the combinatorial use of shRNAs against both HBV and TGF- β in alleviating liver fibrosis [44, 45]. The mechanisms through which RNAi protects against HBV are unclear, and the inhibiting or activating

responses of host factors remains elusive. Isobaric tags for relative and absolute quantification (iTRAQ) has been widely applied to identify differentially expressed proteins in many diseases [46-48] including liver fibrosis [49, 50]. As a potent new technique in comparative proteomics analysis, iTRAQ has relatively high sensitivity and allows the determination of diverse proteins compared with traditional proteome approaches [51].

In this study, we analyzed liver proteins in shRNA-treated mice using iTRAQ-based quantitative proteomics in order to identify differentially expressed proteins and to elucidate the therapeutic mechanisms of liver fibrosis.

Results

Baseline characteristics of the study mice

Our previous study demonstrated that co-administration of shRNAs targeting TGF- β and HBV decreased HBV antigens, HBV DNA, and liver fibrosis markers in the serum and livers of HBV-replicated mice [44]. In order to explore the mechanisms underlying the antiviral and antifibrotic effects, AAVdual-shRNA and AAVshRNA-TGF- β co-injection was evaluated. HBV(+) and HBV(-) mice were used as positive and negative controls, respectively. All three AAVshRNA-treated mice showed lower HBsAg and HBV DNA levels in the serum compared with that in untreated mice (Table 1). HBsAg and HBcAg levels were significantly decreased in the livers of AAVshRNA-treated mice, as demonstrated by IHC staining (Figure. 1).

Collagen levels were significantly decreased in the livers of treated mice compared with those in HBV(+) mice (Figure. 2a, Table 1). Total collagen was also quantitatively assessed using hydroxyproline assays; lower collagen levels were observed in the livers of treated mice and HBV(-) mice than in those of HBV(+) mice (Table 1). Masson staining and Sirius Red staining showed that the percentages of collagen deposition in hepatocytes were decreased by approximately 67.71% and 80.01%, respectively, after treatment. Collagen I and collagen III levels in serum were also significantly reduced in the treated group compared with that in HBV(+) mice (Table 1).

Next, we detected the expression of α -SMA, a marker of fibrosis, in the liver by IHC staining (Figure. 2a) and western blotting (Figure. 2d). As indicated by IHC staining, α -SMA expression was markedly reduced in the treated group compared with that in the HBV(+) group (Figure. 2a). The percentage of α -SMA expression was decreased by over 45% in the livers of treated mice compared with that in HBV(+) mice, as demonstrated by western blotting (Figure. 2d). Taken together, these data indicated that the mouse model in this study was appropriate.

Proteomic analysis of shRNA-treated HBV-replicated mice by iTRAQ-based quantitative proteomics

Next, we investigated differentially expressed proteins and potential pathways for attenuating liver fibrosis using iTRAQ-based quantitative proteomics by comparing these three groups of mice in order to

elucidate the potential antifibrotic mechanisms. AAVdual-shRNA-and AAVshRNA-TGF- β -treated groups were analyzed by iTRAQ-based quantitative proteomics, as shown in the flowchart in Figure. 3a. HBV(+) mice were used as a positive control, and HBV(-) mice were used as a negative control. In total, 2743 proteins were identified in all groups (Figure. 3b, c). Notably, 76 upregulated and 122 downregulated proteins were found in the treated group compared with that in the HBV(+) group (Additional file 1: Table S2). Sixty-one proteins were upregulated, and 134 proteins were downregulated in HBV(+) mice compared with that in HBV(-) mice (Additional file 1: Table S3). We also evaluated the differentially expressed proteins in all three groups using Venn-Euler diagrams (Figure. 3d) and found 41 upregulated and 15 downregulated proteins in the treated group versus the HBV(+) group compared with the HBV(+) group versus the HBV(-) group. Two proteins were upregulated in both comparisons, and two proteins were downregulated in both comparisons (Figure. 3d; Additional file 1: Table S4).

In order to obtain an overall functional view of the differentially expressed proteins, we used GO functional annotations and KEGG metabolic pathway analyses. Comparison of the treated group and HBV(+) group revealed enrichment of 2185 BPs; 321 of these BPs were significant according to analysis of *P* values. Additionally, 89 CCs were significantly altered among 337 enriched CCs, and 484 MFs were enriched, among which 144 MFs were significant. Seventeen KEGG terms among 99 enriched KEGG terms were significant (Figure. 4a). In order to clarify the functions and features of the identified proteins, we annotated protein functions and features based on GO and KEGG analyses. An overview of the GO analysis is shown in Figure 4b. There were 10 distinctly enriched categories of BPs, CCs, and MFs. The top proteins enriched in BPs were involved in organ-nitrogen compound metabolic process (45%), and some proteins enriched in BPs were related to liver fibrosis, e.g., lipid metabolic process (11%), oxidation-reduction (11%), response to oxidative stress (5%), negative regulation of cell adhesion (4%), and cellular oxidant detoxification (2%; Additional file 1: Figure S1 a). The main MF category of enriched proteins was cytoplasm (85%). Proteins involved in hepatic fibrosis and oxidative stress were also observed in MFs, including adherent junction (7%), endoplasmic reticulum membrane (7%), complex of collagen trimers (2%), and the TRAF2-GSTP1 complex (1%; Additional file 1: Figure S1 c). Proteins enriched in CCs were involved in nucleic acid binding (40%), hydrolase activity (8%), oxidoreductase activity (4%), organic acid binding (3%), transferase activity (3%), vitamin binding (2%), and vitamin B6 binding (2%; Additional file 1: Figure S1 c).

These proteins were also mapped to KEGG pathways based on their KEGG gene IDs. There were eight KEGG pathways presented, including metabolic pathways (14%), ribosome (13%), PPAR signaling pathway (6%), chemical carcinogenesis (3%), protein digestion and absorption (2%), protein export (1%), tryptophan metabolism (2%), and valine, leucine, and isoleucine degradation (2%; Figure. 4c). The significant ($P < 0.01$) pathways were ribosome, PPAR signaling pathway, and chemical carcinogenesis (Additional file 1: Figure S2 a).

To clarify the functional relationships of the identified proteins, a PPI network was created using Omicsbean. In the PPI network, GSTP1, which participated in glutathione metabolism, chemical carcinogenesis, and metabolism of xenobiotics by cytochrome P450, and ribosomal proteins, including

Rpl13, Rpl37, and Rpl27a, were upregulated. Additionally, FABP1, ME1, and ACAA1, which were relevant to the PPAR signaling pathway and metabolic pathways, were downregulated in the treatment group compared with that in HBV(+) mice (Figure. 4d and Additional file 1: Figure S2 b).

Verification of proteins associated with oxidative stress and PPAR signaling pathway by western blotting

In order to identify the therapeutic mechanisms of liver fibrosis by AAVshRNA treatment, we next focused on differentially expressed proteins related to oxidative stress, the PPAR signaling pathway, lipid metabolism, and inflammation, which are involved in hepatic fibrosis. Indeed, in our previous study, oxidative stress was found to play an important role in liver fibrosis [45]. Additionally, differentially expressed proteins related to oxidative stress, including GSTP1 and PRDX1, were identified by iTRAQ-based quantitative proteomics. Thus, in order to verify changes in oxidative stress after treatment, we evaluated the expression of GSTP1, PRDX1, and TGF- β , which are involved in oxidative stress and the redox imbalance, by western blotting (Figure. 5). GSTP1 (Figure. 5a) was significantly upregulated in the treated group compared with that in the HBV(+) group (increased 1.57-fold) and was significantly downregulated in HBV(+) mice compared with that in HBV(-) mice (decreased 0.54-fold). PRDX1 (Figure. 5b) and TGF- β (Figure. 5c) were significantly downregulated in the treated group compared with that in the HBV(+) group (decreased 0.35- and 0.74-fold, respectively) and were upregulated in the HBV(+) group compared with that in the HBV(-) group (increased 3.31- and 4.20-fold, respectively). Changes in the expression levels of GSTP1 and PRDX1 verified by western blotting were consistent with the alterations found by iTRAQ-based quantitative proteomics analysis.

Bioinformatics analysis showed that the PPAR signaling pathway was activated in the treated group, as demonstrated by downregulation of ACAA1, ME1, and FABP1. Therefore, we next investigated the differential expression of these proteins regulated by PPAR signaling pathway in the liver by western blotting. The three proteins were significantly downregulated to 11.90% (ACAA1; Figure. 5d), 42.50% (ME1; Figure. 5e), and 47.10% (FABP1; Figure. 5f) in the treated group compared with that in the HBV(+) group. In a comparison of the HBV(+) with HBV(-) groups, we found that the expression levels of ACAA1 and FABP1 were not significantly altered, whereas ME1 was significantly upregulated (increased 1.43-fold; Figure. 5e).

PPAR- γ played key roles in activating PPAR-signaling pathway

There are three different isoforms of PPARs, i.e., PPAR- α , PPAR- β/δ , and PPAR- γ [54]. PPAR- α is mainly expressed in the liver, and PPAR- γ is expressed in adipose and liver tissues. Therefore, we evaluated PPAR- α and PPAR- γ expression by western blotting. The results showed that PPAR- α expression was not altered in all three experimental groups. PPAR- γ was significantly upregulated by 3.20-fold in the livers of treated mice compared with those of HBV(+) mice; however, no significant changes were observed in the livers of HBV(+) and HBV(-) groups (Figure. 6). These findings suggest that PPAR- γ may play an important role in activating the PPAR signaling pathway following AAVshRNAs treatment and that PPAR- α may not have an important role as PPAR- γ in activating the PPAR signaling pathway.

AAVshRNA attenuated NF- κ B P65 phosphorylation in the liver and decreased IL-6 secretion into the serum.

H&E staining was used to investigate the pathological process of liver fibrosis. Although most hepatocytes appeared histologically normal in all three groups, some hepatic necrosis was observed at 6 months in HBV(+) mice (Figure 7A). NF- κ B is a key mediator of inflammatory signaling and plays important roles in liver fibrogenesis[38]. Thus, we then examined the levels of NF- κ B p65 and phosphorylated NF- κ B p65 in vivo and in vitro using western blotting. The levels of phosphorylated NF- κ B p65/NF- κ B p65 were markedly reduced in the treatment group compared with that in the HBV(-) group in livers and in LX-2 cells after transfection with AAVshRNA; 90.5% and 69% decreases were observed in vivo and in vitro after treatment, respectively (Figure. 7b). The expression of the inflammatory factor IL-6 was also measured by ELISA in serum. IL-6 levels in the serum were decreased by over 92% after treatment (Figure. 7c).

Discussion

Chronic HBV infection is a major health problem in developing countries, including China, and up to one-third of chronically HBV-infected individuals will progress to fibrosis, cirrhosis, and even HCC [55-57]. Liver fibrosis involves inflammation induced by a vicious circle of hepatic damage, driving HSC activation and worsening liver damage [9, 10]. Liver fibrosis is a reversible process that represents the pivotal early stage of hepatic cirrhosis [58], and few therapies for liver fibrosis have been developed. Thus, it is necessary to elucidate the mechanisms of hepatic fibrosis and develop new medicines for blocking and reversing hepatic fibrosis. Our previous study showed that AAVshRNA had anti-hepatic fibrosis effects in HBV-replicated mice with liver fibrosis [44]. Moreover, fibrotic markers, including α -SMA, collagen I, and collagen III, were significantly reduced. However, the mechanisms mediating the antifibrotic effects of AAVshRNA are still unclear. In this study, ITRAQ-based quantitative proteomics was used to elucidate the antifibrotic mechanism of AAVshRNA. Through a comprehensive analysis comparing the treatment group and HBV(+) mice, we found that ribosomal proteins, downstream proteins of the PPAR signaling pathway, and inflammation- and oxidative stress-related proteins were significantly enriched in the AAVshRNA-treated group. In order to elucidate the mechanisms of liver fibrosis, we investigated the involvement of oxidative stress, the PPAR signaling pathway, and inflammation, which are closely associated with liver fibrosis.

Previously studies have suggested that TGF- β , GSTP1, and PRDX1 are correlated with oxidative stress or ROS imbalance [18, 59-61]. In this study, we also found that these proteins were altered in treated mice compared with that in HBV(+) mice. Many reported have demonstrated that TGF- β can inhibit the antioxidant system and cause oxidative stress or redox imbalance [18, 62-64]. Additionally, PPAR- γ activation can block the TGF- β signaling pathway [65]. Hence, disruption of TGF- β expression can relieve oxidative stress. In this study, reduction of TGF- β expression was observed following treatment with AAVshRNA-TGF- β by direct inhibition of TGF- β mRNA at the transcript level, resulting in upregulation of PPAR- γ . The findings indicated that AAVshRNA treatment alleviated oxidative stress by reducing TGF- β

expression. PRDXs, as redox-regulating proteins, function to eliminate various ROS and maintain cellular redox homeostasis [66]. PRDX1 can be easily over oxidized on its catalytically active cysteine upon stimulation with various stimuli [60]. PRDX1 was significantly upregulated in HBV(+) mice compared with that in HBV(-) mice and was downregulated after treatment, indicating that oxidative stress was reduced. As an important phase II enzyme, GSTP1 can protect cells from oxidative stress in human cancers [59, 67]. In accordance with a previous study, we found that GSTP1 was elevated to alleviate oxidative stress and played a critical role in antioxidant defense after AAVshRNA treatment. Collectively, these findings showed that AAVshRNA treatment could prevent oxidative stress through suppressing the oxidative stress inducers TGF- β and PRDX1 and enhancing GSTP1 expression.

In the PPI network, proteins up- or downstream of the PPAR signaling pathway (including ACAA1, ME1, and FABP1) were found to be regulated, suggesting activation of the PPAR signaling pathway. Notably, FABP1 and ME1 were downregulated in the PPAR signaling pathway, as demonstrated by KEGG analysis. These proteins also played pivotal roles in fatty acid synthesis and transport. ACAA1 is broadly expressed in humans and animals and can catalyze free cholesterol and long-chain fatty acids to synthesize esterified cholesterol [68]. ACAA1 is also a marker of beta-oxidation [69, 70]. ME1 is the cytoplasmic component of the NADPH pool and is used by fatty acid synthase as a primary lipogenic enzyme. ME1 is also dysregulated in many types of cancers and is involved in tumorigenesis and metastasis [71, 72]. FABP1 is a liver-specific fatty acid-binding protein with key roles in intracellular metabolism [73]. Overexpression of FABP1 significantly promotes hepatocyte fatty acid uptake [74], de novo lipogenesis [75], and VLDL secretion [73, 76]. In addition, knockdown of FABP1 significantly suppressed lipid accumulation in hepatocytes [76] and markedly reduced liver weight and hepatic triacylglycerol accumulation [75]. Consistent with previous research, we found that ACAA1, ME1, and FABP1 were downregulated in treated mice compared with that in HBV(+) mice. These results suggested that AAVshRNA treatment inhibited lipogenesis and improved lipid metabolism. Hepatocyte steatosis was observed in HBV(+) mice, consistent with our previous study [77], and was alleviated after AAVshRNA treatment [44]. These findings suggested that AAVshRNA alleviated hepatocyte steatosis and liver fibrosis by decreasing hepatocyte fatty acid uptake and de novo lipogenesis via attenuation of FABP1 and ME1 expression.

PPAR- γ has broad anti-inflammatory effects and plays important roles in controlling fibrogenesis and reducing oxidative stress [31, 33, 54]. Our data indicated that AAVshRNA activated the PPAR signaling pathway by upregulating PPAR- γ expression directly, resulting in decreased expression of liver fibrosis markers (α -SMA and ECM) and inflammatory factors (TGF- β and IL-6), consistent with previous studies. Recent investigations have shown that NF- κ B is a crucial mediator of inflammatory signals and that activation of NF- κ B promotes liver fibrogenesis [37]. Therefore, inhibition of the NF- κ B pathway may have therapeutic effects on liver fibrosis. In this study, we also found that NF- κ B p65 phosphorylation was inhibited in treated mice compared with that in HBV(+) mice and in cells transfected with AAVshRNA. Overall, these data suggested that AAVshRNA inhibited liver fibrosis by blocking the NF- κ B pathway.

Based on these findings, we proposed an antifibrotic model for AAVshRNA (Figure. 8). AAVshRNA induced PPAR- γ expression and inhibited TGF- β expression and NF- κ B phosphorylation. TGF- β was downregulated after AAVshRNA treatment in HBV-replicated mice, leading to relief of oxidative stress by upregulation of GSTP1 and downregulation of PRDX1. TGF- β was also downregulated owing to upregulation of PPAR- γ . Upregulation of PPAR- γ resulted in activation of the PPAR- γ signaling pathway. The PPAR signaling pathway influenced lipid metabolism by decreasing the expression of FABP1 and ME1 and reducing hepatocyte steatosis. At the same time, upregulation of PPAR- γ inhibited inflammation by blocking the NF- κ B signaling pathway through decreasing NF- κ B p65 phosphorylation.

Conclusions

Taken together, our findings suggested that activation of the PPAR signaling pathway played key roles in curing hepatic fibrosis through alleviating oxidative stress and inflammation and improving lipid metabolism, resulting in inhibition of liver fibrosis by blocking α -SMA, collagen I, and ECM production.

Methods

Animal study

Normal C57BL/6 mice (aged 6–8 weeks, male; Vitalriver, Beijing, China) were housed and maintained at the Laboratory Animal Facility of the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Science, Beijing. The protocols for the care and use of laboratory animals were approved by the Institutional Animal Care and Use Committee at the Chinese Academy of Medical Science, and all animal care procedures and experiments were performed in accordance with these protocols. AAV8-HBV1.2 vector (2×10^{11} vector genome equivalents [vg]) was injected into mice via the tail vein to construct an HBV persistent replication model. Serum samples were obtained by collection of blood from the tail vein into heparinized capillary tubes using standard methods 1 month after injection and then subjected to enzyme-linked immunosorbent assays (ELISAs) and quantitative polymerase chain reaction. HBV surface antigen (HBsAg)-, HBV e-antigen (HBeAg)-, and HBV DNA-positive mice were designated as HBV(+) mice and injected with AAVshRNA1+3 and AAVshRNA-TGF- β or phosphate-buffered saline (PBS). Normal C57BL/6 mice were also injected with PBS as a negative control and designated as HBV(-) mice. All mice were sacrificed at 6 months after injection. Serum and liver samples were collected and frozen at -80°C in a freezer or liquid nitrogen.

At the end of the experiment, mice were anesthetized with 2.5% avertin and perfused with cold PBS (pH 7.4) transcardially, followed by 4% paraformaldehyde in PBS (0.1 M, pH 7.4). Livers were then collected for immunohistochemical (IHC) analysis. Intrahepatic HBV core antigen (HBcAg) and HBsAg were evaluated by IHC staining of OCT-embedded tissues with rabbit anti-HBc and anti-HBs antibodies (Dako, Carpinteria, CA, USA), respectively, and developing with the Envision HRP (diaminobenzidine) system

(Dako). Liver sections were examined with light microscopy after standard hematoxylin and eosin (H&E) and Masson's trichrome staining. Sirius red staining of liver sections was observed by polarizing microscope. Total collagen in the liver was determined using a Hydroxyproline Colorimetric Assay Kit (BioVison, Milpitas, CA, USA).

Protein preparation and iTRAQ labeling

Total protein extraction was performed using a kit (FOCUS- Mammalian Proteome; G-Biosciences, USA) in accordance with the manufacturer's instructions. Protein samples were stored at -80°C for proteomic analysis and western blotting. The iTRAQ method was described previously [52]. Briefly, total protein concentrations were determined using an EZQ Protein Quantitation Kit (Invitrogen, USA), and protein samples from treated mice, HBV(+) mice, and HBV(-) mice were reconstituted in dissolution buffer, denatured, reduced, and trypsinized. Next, tryptic digests of the samples were labeled with iTRAQ reagents (Table S1). All samples were balanced, mixed, and pre-separated for liquid chromatography (LC)-mass spectrometry (MS)/MS analysis.

Nano-LC-MS/MS analysis

LC-MS/MS analysis was performed with an Easy-nLC1000 (Thermo Fisher Scientific, USA) and Q ExactiveMS (Thermo Fisher Scientific). A reversed-phase ReproSil-PurC18-AQ column (column: $3\text{ }\mu\text{m}$, $120\text{ }\text{\AA}$, $100\text{ }\mu\text{m}\times 10\text{ cm}$) was used to separate the peptides at a flow rate of 600 nL/min . The LC linear gradient elution was performed from 6% to 9% B for 15 min, 9% to 14% B for 20 min, 14% to 30% B for 60 min, 30% to 40% B for 15 min, and 40% to 95% B for 3 min, followed by elution with 95% B for 7 min. A precursor scan was performed using an Orbitrap instrument by scanning from $m/z\text{ }300\text{--}650$ for detection with Q ExactiveMS. The MS resolution was 60000 at $m/z\text{ }400$. The parameters of MS/MS settings were as follows: the product ion scan range started at $m/z\text{ }100$; the activation type was CID; the minimum signal required was 1500; the isolation width was 3; the normalized collision energy was 40; the default charge state was 6; the activation Q was 0.25; the activation time was 30 s; and the data dependent MS/MS was up to the top 5 most intense peptide ions from the preview scan in the Orbitrap.

Functional Analysis of differentially expressed proteins

In order to reduce false positives of differentially expressed proteins, an additional cut off of fold change greater than 1.30 or less than 0.77 ($1/1.3$) was exploited for all iTRAQ ratios [53]. Proteins with iTRAQ ratios greater than 1.30 or less than 0.77 were considered upregulated or downregulated, respectively. Gene ontology (GO) annotations, pathway enrichment, and protein-protein interaction (PPI) networks for all the identified proteins and differentially expressed proteins were evaluated with Omicsbean

(<http://www.omicsbean.cn>). GO annotations were classified into three major categories, including biological processes (BPs), cell components (CCs), and molecular functions (MFs). Pathway enrichment analysis was performed with Kyoto Encyclopedia of Genes and Genomes (KEGG) mapping. PPI networks were applied to obtain key nodes, such as degree centrality, betweenness, closeness, and cluster coefficient.

Immunoblotting analysis

For immunoblotting, 10 µg protein from liver tissue was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4–12% gels. The separated proteins were blotted onto polyvinylidenedifluoride membranes, and the membranes were then washed with TBST and then incubated with blocking buffer containing 5% skimmed milk in TBST for 2 h at 25 °C. Membranes were washed again with TBST and incubated overnight at 4°C with primary antibodies diluted in TBST. The primary antibodies were rabbit anti-mouse antibodies targeting glutathione S-transferase Pi 1 (GSTP1; Proteintech Group, Inc., Rosemont, IL, USA), peroxiredoxin-1 (PRDX1; Proteintech Group), acetyl-CoA acyltransferase 1 (ACAA1; Proteintech Group), malic enzyme 1 (ME1; Proteintech Group), fatty acid binding protein 1 (FABP1; Proteintech Group), PPAR-α (Proteintech Group), PPAR-γ (Proteintech Group), α-SMA (Abcam, Shanghai, P.R.China), TGF-β (Proteintech Group), NF-κBp65 (Cell Signaling Technology, Inc., Danvers, MA, USA), phospho-NF-κBp65 (ser468; Cell Signaling Technology, Inc.), and glyceraldehyde 3-phosphate dehydrogenase (Abcam). The membranes were then incubated with goat anti-rabbit secondary antibodies (Thermo Fisher Scientific) for 1 h at room temperature. Finally, the signal was visualized using an electrochemiluminescent reagent kit (Millipore Corporation, Billerica, MT, USA), and blots were imaged using X-ray film.

Cell lines

LX-2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution at 37°C in a humidified incubator with 5% CO₂. LX-2 cells were seeded into 6-well plates at 4×10⁵ cells/well and cultured. pSSV9-HBV1.2 was transfected into LX-2 cells using Lipofectamine 2000 (Thermo Fisher Scientific) with or without pAAV-shRNAs (3µg) according to the manufacturer's instructions. Lipofectamine was used as a negative control. The supernatants and transfected cells were collected 72 h after transfection and subjected to protein extraction with RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific). The concentration of protein was determined with a Thermo Scientific Pierce BCA Protein Assay Kit.

Statistical analysis

The data are reported as means ± standard deviations. One-way analysis of variance (GraphPad Prism 5.0) was used to determine statistically significant differences between groups. Differences with *P* values

of less than 0.05 were considered statistically significant.

Abbreviations

AAV: adeno-associated virus; shRNA: short hairpin RNA; HBV: hepatitis B virus; TGF: transforming growth factor; iTRAQ: isobaric tags for relative and absolute quantitation; PPAR: peroxisome proliferator-activated receptor; HCC: hepatocellular carcinoma; ECM: extracellular matrix; HSC: Hepatic stellate cells; α -SMA: α -smooth muscle actin; ROS: reactive oxygen; NF- κ B: nuclear factor- κ B; IL-6: interleukin-6; RNAi: RNA interference; IHC: immunohistochemistry; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; CC: cell component; MF: molecular function; BP: biological process; TRAF2-GSTP1: Tumor necrosis factor receptor associated factor 2-glutathione S-transferase Pi; PPI: protein-protein interaction; GSTP1: glutathione S-transferase Pi 1; Rpl: ribosome large subunit protein; FABP1: fatty acid binding protein 1; ME1: malic enzyme 1; ACAA1: acetyl-CoA acyltransferase 1; PRDX1: peroxiredoxin-1; H&E staining: hematoxylin-eosin staining; ELISA: enzyme linked immunosorbent assay; VLDL: very low density lipoprotein; PBS: phosphate buffer saline; OCT: optimal cutting temperature compound; TBST: Tris-buffered saline tween-20; LC-MS/MS: liquid chromatography-mass spectrometry/ mass spectrometry; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Declarations

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Authors' contributions

LY, WL and CZ conceived and designed the experiments. LY, JC, LS, and TC performed the experiments. LY analyzed the data. LY and WL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study of HBV-related liver fibrosis in mice was performed in accordance with the Guide for the Care and Use of Laboratory Animals, which was approved by the Institutional Animal Care and Use Committee at the Chinese Academy of Medical Science. Mice were maintained at the Laboratory Animal Facility of the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Science, Beijing.

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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Tables

Table 1. Baseline characteristics of mice used in this study.

Group	Sex (Male/Female)	HBsAg serum (IU/mL)	HBV-DNA serum (copies/mL)	HBV-DNA liver (copies/g)	Collagen liver (μ g/mg)	Collagen I serum (pg/mL)	Collagen III serum (pg/mL)
Treated N=3	M	125.80	1.80×10^4	2.01×10^8	107.48	141.34	0.51
	M	383.70	2.51×10^4	2.00×10^8	112.24	159.45	0.75
	M	0.40	1.04×10^4	1.60×10^8	104.55	143.51	0.62
HBV(+) N=3	M	>2500.00	1.70×10^5	2.15×10^8	241.29	332.12	1.31
	M	>2500.00	4.24×10^4	4.81×10^8	236.29	349.49	1.45
	M	>2500.00	5.02×10^4	5.01×10^8	375.75	365.75	1.75
HBV(-) N=3	M	-	-	-	95.50	131.14	0.30
	M	-	-	-	100.21	145.49	0.52
	M	-	-	-	94.01	132.42	0.27

Figures

Figure 1

AAV shRNA treatment decreased HBsAg and HBcAg in hepatocytes from HBV-replicated mice. Liver samples from mice treated with AAV shRNA were collected at 6 months after injection. Liver sections were fixed and stained for HBsAg and HBcAg using immunohistochemistry. Scale bar: 100 μ m.

Figure 2

AAV shRNA treatment attenuated collagen deposition and liver fibrosis in HBV-replicated mice. Mice received AAV-shRNA treatment and were euthanized at 6 months after vector administration. Collagen deposition and collagen fibers were examined by Masson's trichrome and Sirius red staining of liver sections, respectively. Masson staining and Sirius Red staining were observed by light microscopy. Sirius Red stain was also observed by polarized optical microscopy (a). α-SMA was detected by IHC (a) and western blotting (d). Quantification of collagen deposition by Masson staining (b) and Sirius Red staining (c) was performed using Image-Pro Plus software, and Image J software was used to quantify α-SMA in western blotting (d). Scale bars in Masson and Sirius Red staining: 250μm. Scale bar in IHC of α-SMA: 20μm.

Figure 3

Differentially expressed proteins were identified by the iTRAQ method. (a) Schematic flowchart of the iTRAQ method. Total proteins were identified in the treated, HBV(+) (b), and HBV(-) groups (c). Up- and down-regulated proteins were identified, and fold changes of over 1.3 were depicted in Venn-Euler diagrams (d).

Figure 4

Bioinformatics analysis of the treated group versus the HBV(+) group. (a) Statistical summary of bioinformatics analysis. (b) Biological processes, cell components, and molecular functions by GO analysis. (c) Global view of the KEGG pathways affected. (d) PPI analysis of differentially expressed

proteins. Squares indicate GO/KEGG terms, circles indicate proteins/genes, red circles indicate upregulated proteins, and green circles indicate downregulated proteins.

Figure 5

Oxidative stress was alleviated and downstream proteins in the PPAR signaling pathway were altered by AAVshRNA treatment. The differentially expressed proteins GSTP1 (a), PRDX1 (b), and TGF-β (c) were identified and confirmed by western blotting. Downstream proteins in the PPAR signaling pathway, including ACAA1 (d), ME1 (e), and FABP1 (f), were downregulated in the treated group compared with that in the HBV(+) group.

Figure 6

PPAR- γ rather than PPAR- α played a key role in activating the PPAR signaling pathway. Mice received AAVshRNA treatment and were sacrificed at 6 months after injection. Total protein was extracted from liver samples. The expression of PPAR- α (a) and PPAR- γ (b) was detected by western blotting.

Figure 7

AAVshRNA treatment improved liver tissue histology and alleviated inflammation by attenuating the NF-κB signaling pathway and reducing serum IL-6 levels. Mice received AAVshRNA treatment, and liver samples were collected at 6 months after injection. Liver sections stained with hematoxylin and eosin (a). LX-2 cells were transfected with pSSV9-HBV with or without AAVshRNA. Phospho-NF-κB (Ser468)/NF-κB p65 levels were evaluated (b). IL-6 concentrations in serum were determined by ELISA (c).

Figure 8

A proposed model showing the mechanism of reduced oxidative stress, inflammation, and PPAR-γ signaling activation, resulting in antifibrotic effects of AAVshRNA treatment. Red color indicates upregulated proteins and positive correlations. Blue color indicates downregulated proteins and negative correlations.

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