Hepatitis C Virus nonstructural protein 4B induces lipogenesis via Merlin-Hippo pathway

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Abstract
Hepatitis C virus (HCV) infection is often associated with hepatic steatosis, even hepatocellular carcinoma. Nonstructural protein 4B (NS4B) a highly hydrophobic non-structural protein, induces lipogenesis, but the underlying mechanism remains incompletely understood. In the present study, NS4B expression in Huh7.5 cells could reduce moesin-ezrin-radixin-like protein (Merlin, NF2 gene coding protein) expression, Yap phosphorylation, and increase AKT phosphorylation and sterol regulatory element-binding proteins (SREBPs) expression, which regulate lipogenic gene expressions including fatty acid synthase (FAS). In Huh7.5 cells, Merlin silencing reduced Yap phosphorylation, AKT phosphorylation, and especially SREBPs levels lead to reduction of lipid drop synthesis. In addition, HCV NS4B infected Huh7.5 cells exhibited lipid droplets, but lipid droplets significantly reduced after Merlin over-expressed. In conclusion, these results indicated that NS4B may play an important role in HCV-associated liver pathogenesis via Merlin-Hippo signal pathway.

Introduction
Chronic liver disease caused by virus infection distribute widely in the word, and always associated with high morbidity and mortality, one of the leading causes is HCV infection(1). The host liver can be damaged progressively once infected by HCV and the infection is persistent(2,3). Host factors synthesis can be promoted obviously once infected by HCV for virus propagation, such as cellular lipid generation and metabolism(4,5). It has been clearly shown that lipid generation and metabolism can be altered by HCV in infected hepatocytes(4,6,7), and it is the main reason why hepatic steatosis always found in HCV infected patients. Li, et al.(8) reported that HCV exploited intrinsic response and hijacked lipid metabolism through the interaction between HCV 3-untranslated region (3-UTR) and DDX3X, and in turn activated IKKα, which enhanced core-associated lipid droplet formation for viral assembly. The mechanistic basis of these functional effects still needs far more investigations to be clear.. Although hepatic steatosis always can be found in HCV infected patients(9,10), and we also can be sure that lipid generation and metabolism is strongly associated with HCV infection, the molecular mechanisms that related with lipogenises during HCV infection are poorly understood. Generaly speaking, HCV genome encodes a polyprotein of more than 3,010 amino acids that is
cleaved at endoplasmic reticulum (ER) by host and viral proteases, including structural and nonstructural proteins. NS4B, one of nonstructural proteins, 27 kDa, a hydrophobic protein locating through the ER membrane(11,12) with N-terminal tail, functions remain confused. Elazar, et al.(13) found that membrane anchoring could be mediated by N-terminal 26 residues of NS4B, which were important for HCV replication in vitro experiment. Furthermore, NS4B also be related with NIH-3T3 cells transformation with Ras(14) or without Ras(15).

HCV infection can significantly affects lipid-related metabolism in vivo including lipids and circulating lipoprotein, which has been indicated by clinical research evidence(16). The liver is the main center of lipid production via uptaking lipoproteins and deliver lipids to other organs(17). HCV infected patients often suffer from type II diabetes cause the insulin resistance, one of pathological features in HCV infected patients. Furthermore, hepatic fibrosis, steatosis, hepatocellular carcinoma and resistance to anti-viral treatment(18) also found to be related with insulin resistance. Recent evidences showed that hepatic steatosis can be induced by HCV core protein. They found that the microsomal triglyceride transfer protein activity and very low density lipoprotein secretion(19) were inhibited by HCV core protein, and then expression and transcriptional activity of peroxisome proliferators-activated receptor (PPAR)α(20) was impaired,resulting in the SREBP1 and PPARα(21) activation.

SREBPs are endoplasmic reticulum binding transcription factors that up-regulate the activation of enzymes that facilitate the cholesterol and fatty acids synthesis, and lipoproteins cellular uptake. Park and coworkers demonstrated that HCV NS4B modulated SREBP1 via the AKT pathway(22). Wu, et al. (23) demonstrates that HCV NS4B protein activates PI3K/AKT pathway via up-regulating Snail in HCC and inhibiting Hippo signal pathway.

In this present study, our findings showed that NS4B inhibited Merlin and Yap phosphorylation, and also up-regulated AKT phosphorylation to lead to lipogenesis. The results may provide a novel mechanism of hepatic steatosis associated with HCV infection.

Materials And Methods

Cells and plasmids

Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) was used to maintain
293T and Huh7.5.1 cells, 5% CO2 incubator and temperature was set to 37°C to culture cells.
pCDNA3.1-NS4B was kindly send by Yi-Zheng (Huadu District People's Hospital, China). Full-length Merlin sequence was inserted into pFLAG-CMV2 vectors to construct pFLAG-CMV2-Merlin. PCR and DNA sequencing detection methods were used to testify the integrity and correctness of recombinant plasmids. The plasmids were transfected into cells by lipofectamine-2000 (Invitrogen, Karlsruhe, USA) according to manufacturers' instructions when the cells confluence approximately 80%.

**Western blotting analysis**
Cells were lysed in cell lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, and protease inhibitor mixture) for 20 min on ice. ECL kit (Abcam) was purchased to detecte proteins. The equivalent amount of protein was electrotransferred to the nitrocellulose membrane with 10% sds-page treatment. Phosphate-buffered saline (PBS) including 5% nonfat dry milk was used to block the membrane for 1 h and then hatch 2 h at room temperature with one of following antibodies: anti-NS4B antibody, anti-AKT and anti-P-AKT antibody (Abcam), anti-Merlin, anti-Yap, anti-P-Yap, anti-SREBP-1 antibody (Santa Cruz Biotechnology), anti-GAPDH and anti-FLAG antibody (Sigma). Membrane was washed twice in Tris-buffered saline/Tween, and then was hatched with either horseradish peroxidase-conjugated goat anti-rabbit antibody or goat anti-mouse antibody (Sigma) in Tris-buffered saline/Tween for 1 h at room temperature.

**siRNA transfection**
293T and Huh7.5 cells were seeded on 12-well plates at 5x10^5 cells per well separately and when the cells confluence approximately 60%, cells were transfected with siRNAs of NF2 (Si-NF2) and siRNAs control of NF2 (Si-control) using RNAiMAX (Invitrogen). Further treatments or tests were typically performed 48 or 72 h after siRNA transfection.

**Oil red O test**
When cultured cell density reached 5-6x10^5 cells/ml, medium drained and cells were washed twice with 0.01 M PBS, The cells were fixed with phosphate buffer containing 10% formaldehyde for 10 mins, rinsed with PBS for 1min and 60% isopropanol for 15 seconds. The cells were stained with filtered Oil red O working solution with light avoid for 1min at 37°C, rinsed with 60% isopropanol for
15 seconds and PBS 3 times for 3 min. Cells stained with hematoxylin counterstain for 1 min and rinsed with PBS 3 times for 3 min. Tablet was sealed and observed under microscope.

**Statistical analysis**

Strip grayscale analysis (Quantity one) was processed to analyse the protein western blot results. All experimental data acquired the mean of three independent experiments (n=3) and expressed as the mean ± standard error. Tukey's test was used to determine difference between different groups. All data were analyzed with SPSS(22.0) software. P<0.05 was considered statistically difference.

**Results**

1. **NS4B regulates Hippo pathway via NF2**

To confirm the relationship between NS4B and Hippo signal pathway, NS4B plasmids were transfected into Huh7.5 cells separately, cells were collected and subjected to RT-PCR and western blot assays. To further confirm the results, we transfected different amount of NS4B plasmids (0.5 μg, 1 μg, 1.5 μg) into Huh7.5 cells and collected cells at different time points (24 h or 48 h later). The results showed that NF2 mRNA level is obviously decreased after NS4B plasmid transfected into Huh7.5 24 h and 48 h (Fig.1 A), which indicated that NS4B could mediate Merlin expression from gene level. Next, western blot assays verified the gene tests. As shown in Fig.1 B-D, Merlin expression was downregulated and Yap phosphorylation (P-Yap) was up-regulated on 24 h and 48 h points after the same amount of NS4B plasmid transfected. At the same time, similar results were found in Huh7.5 cells 24 h later when different amount of NS4B plasmid transfected(Fig.1 E,F,). These results showed that HCV NS4B do mediate Hippo signal pathway.

Furthermore, we also detected some protein expression or phosphorylation of AKT signal pathway. We transfected 0.5 μg or 1 μg pCDNA3.1-NS4B into 293T or Huh7.5 cells separately and take pCDNA3.1 plasmid as control. 24 h later, total AKT, AKT phosphorylation (P-AKT) and NF-κB expression was detected by western blot assays. Results showed that total AKT protein expression rarely changed(Fig. 1G-L), however, P-AKT and NF-κB expression up-regulated obviously(Fig.1 G-L) in 293T or Huh7.5 transfected pCDNA3.1-NS4B.

2. **Merlin mediated AKT signal pathway**
We have found HCV NS4B can regulate Hippo signal pathway and AKT signal pathway, we hypothesized that these two pathways connected here. To test this hypothesis, we transfected different amount of Merlin expression plasmid into Huh7.5 cells and then examined the protein expression and phosphorylation levels of AKT and NF-κB. Increased expression of Merlin significantly reduced p-AKT and NF-κB, however total AKT expression has no alternation(Fig.2 A-B ). We also downregualted the Merlin expression and examined the protein expression and phosphorylation levels of AKT and NF-κB with siRNA of NF2 gene to explored the connection of Hippo pathway and AKT pathway from the contrary side. We transfected siRNA of NF2 into 293T and Huh7.5 cells at a 50 nM final concentration, 72 h later, protein expression and phosphorylation levels of AKT and NF-κB were examined. The results showed that P-AKT and NF-κB expression levels were up-regulated when Merlin was down-regulated but total AKT expression has no change(Fig.2 C-F ) in 293T or Huh7.5 cells . These results suggested that HCV NS4B mediated AKT signal pathway via inhibiting Merlin-Hippo signal pathway.

3. **NS4B and Merlin induces lipogenesis and SREBP-1 expression**

In order to test whether Merlin has a regulatory effect on lipid metabolism in Huh7.5 cells, we verified by Oil red O tests with high expression of Merlin and silence of NF2 gene in Huh7.5 cells, and high expression of NS4B as control. In Huh7.5 cells, lipid droplets significantly increase after pCDNA3.1-NS4B transfection(Fig.3 B,F), whereas the lipogenesis decreased after Merlin over-expression(Fig.3 C,F). Lipid droplets reduced in cells expressing NS4B after transfection with Merlin palsmid(Fig.3 A,F). However, Merlin silence led to lipid droplets significantly increase compared with cells transfected pCDNA3.1-NS4B(Fig.3 D,F), suggesting NS4B and Merlin mediate lipogenesis oppositely in Huh7.5 cells. To further investigate the mechanism of NS4B induced lipogenesis, we transfected pCDNA3.1-NS4B, pFLAG-NF2 and Si-NF2 into 293T or Huh7.5 cells and examined the protein SREBP-1. The results showed that the protein level of SREBP-1 was significantly up-regualted by NS4B(Fig.3 G-J) and Si-NF2(Fig.3 M,N), and decreased by Merlin(Fig.3 K,L), which consistent with our Oil red O results(Fig.3 A-F). Altogether, these results implied that NS4B works in the context of HCV to trigger lipogenesis via Merlin reduced.
Discussion
Hippo pathway plays a pivotal role in regulating tissue growth size through mediating cell growth, proliferation and apoptosis(24,25). Recently, researchers reported that the effect of Hippo pathway can be found in different cell types including hepatic cells(26). They found that the change of expression and phosphorylation levels of Yap, one of the key substrates of Hippo pathway, would regulate the growth of hepatic tissue directly(27,28), and this is partially why Hippo pathway is one of the important pathway in the mediating mechanism of hepatic cell. Merlin was reported to be a tumor suppressor(29) and Merlin significantly connected with key factors of Hippo pathway(30,31). In this paper we detected Merlin protein expression level in Huh7.5 cells transfected with HCV NS4B plasmid. Our results showed that Merlin was down-regulated(Fig. 1A-F). We speculate that the Hippo pathway probably participates in the mechanism of cell biology affected by NS4B. To identify this hypothesis, we also detected the phosphorylation level of Yap and found that the phosphorylation level of Yap exhibited a positive correlation with NS4B protein level(Fig. 1A-F). With these results, we believed that the Hippo pathway probably was partly affected by NS4B protein. Furthermore, as previous studies demonstrated that HCV control liver size via Merlin protein(32), we speculate NS4B might be a related protein of HCV with liver size, but this hypothesis still needs more researches to be verified.

There is no doubt about HCV is strongly related with lipid accumulation in hepatic tissue. Hepatic steatosis can be found in 40%-86% HCV infected patients. Some studies had reported the correlation between HCV and lipid metabolism in hepatic cells(10,17,21,33,34). NS4B was reported to accelerate the lipid synthesis through AKT pathway(35). In this paper, we checked the effect of NS4B on AKT pathway and the results(Fig. 1G-L) were consistent with the previous reports(35). However, there are no more reports about the mechanism about the effect of NS4B on AKT at present. Interestingly, there are some reports about the correlation of Merlin with AKT pathway(36,37), therefore, we conjectured that Merlin is involved in the effect of NS4B on AKT pathway. To test this hypothesis, we measured the key proteins expression and activity of AKT pathway in Huh7.5 cell line after Merlin being over-expressed or silenced separately. The results showed that the total AKT expression could not be influenced by Merlin(Fig. 2). But the activity of AKT has negative correlation with Merlin.
expression (Fig. 2). These results indicated that Merlin might be one of the upstream regulators of AKT pathway, and NS4B probably mediates AKT pathway via Merlin. The roles of AKT pathway in the synthesis of lipid droplets and Merlin in the synthesis of fatty acid had been confirmed (38). We believed that Merlin was related with lipid droplets synthesis as well. We detected the lipid droplets in Huh7.5 cell line after Merlin being over-expressed or silenced separately. We find that the lipid droplet volume is obviously up-regulated when NS4B over-expressed in Huh7.5 cells (Fig. 3B,F). Interestingly, we also found that the lipid droplet volume is obviously up-regulated when Merlin is silenced (Fig. 3D,F), but the lipid droplet volume is obviously down regulated when Merlin is over-expressed (Fig. 3C,F). At the same time, to confirm this correlation, we measured the lipid droplets related protein, SREBP-1. Results showed that the SREBP-1 protein can be up-regulated in Huh7.5 cells either NS4B within (Fig. 3G-J) or Merlin gene is silenced (Fig. 3M,N). Conversely, SREBP-1 was down-regulated in Huh7.5 cells when Merlin was over-expressed (Fig. 3K,L).

Although the relationship between NS4B and lipid droplets still needs more research and we also realized that it is still too early to draw a final conclusion. We believe that HCV NS4B is one of the related proteins with lipid droplets in Huh7.5 cells and in view of the reported results (39), we speculate the Hippo pathway might be a bridge between NS4B and AKT pathway.

Declarations

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Conflict of interest

The authors declare no conflicts of interest

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.
Authors' contributions
Zhanfeng-Zhang and Yingchun-Zhou designed the study. Zhanfeng-Zhang, Rong-Zhao, Jiukai-Chen, Zaichun-Xie, Zhiyu-Pang performed the experiments. Rong-Zhao analysed the data. Zhanfeng-Zhang prepared the manuscript. All authors have seen and approved the final published version of this manuscript.

Ethics approval and consent to participate
Not applicable.

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Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Figures
Figure 1
Effect of NS4B on proteins expression of Hippo pathway and AKT pathway in 293T or Huh7.5 cells. (A) RT-PCR analysis the mRNA expression of NF2 in control group and NS4B group. The Huh7.5 cells were transfected with 1 μg pCDNA3.1-NS4B plasmid, 24 h and 48 h later, total mRNA was extracted and RT-PCR was performed. Data were presented as mean±SE (n=3). (B,C,D) Huh7.5 cells were transfected with pCDNA3.1 plasmid (control group) and pCDNA3.1-NS4B plasmid (NS4B group), 24 h and 48 h later, the expression of Melrin, P-Yap and Yap in control group and NS4B group. (E,F) Huh7.5 cells were transfected with pCDNA3.1 plasmid (control group), 0.5, 1, 1.5 μg pCDNA3.1-NS4B plasmid respectively, 48 h later, the expression of Melrin, P-Yap and Yap in each group. (G-L) 293T and Huh7.5 cells were transfected with pCDNA3.1 plasmid (control group), 0.5, 1 μg pCDNA3.1-NS4B plasmid respectively, 48 h later, the expression of AKT1/2/3 (total AKT), P-AKT and NF-κB in each group. * indicate significant differences at P<0.05 between two groups.
Figure 2

Effect of Merlin on Key proteins expression of AKT pathway in 293T or Huh7.5 cells.

(A,B)Huh7.5 cells were transfected with pFLAG-CMV2 plasmid (control group), 0.5, 1, 1.5 μg pFLAG-Merlin plasmid respectively, 48 h later, the expression of AKT1/2/3(total AKT), P-AKT and NF-κB in each group. (C-F)293T and Huh7.5 cells were transfected with PBS buffer (Blank), Si-control and Si-NF2 respectively, 48 h later, the expression of AKT1/2/3(total AKT), P-AKT and NF-κB in each group. * indicate significant differences at P<0.05 between two groups.
Lipid metabolism and SREBP-1 expression in Huh7.5 cells affected by NS4B or Merlin. (A-F) Oil red O test confirm the lipogenesis in Huh7.5 cells were transfected with pFLAG-Merlin(C) or Si-NF2(D) or pCDNA3.1-NS4B(B) or co-transfected pCDNA3.1-NS4B and pFLAG-Merlin(A) or Si-control of Si-NF2(E). (G-J) 293T and Huh7.5 cells were transfected with pCDNA3.1 plasmid (control group), 0.5, 1 µg pFLAG-Merlin plasmid respectively, 48 h later, the expression of SREBP-1 in each group. (K-N) 293T and Huh7.5 cells were transfected with pFLAG-CMV2 plasmid (control group), 0.5, 1, 1.5 µg pCDNA3.1-NS4B plasmid respectively, 48 h later, the expression of SREBP-1 in each group. * indicate significant differences at P<0.05 between two groups.