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***Basic Study***

**Hepatitis C virus core protein-induced miR-93-5p inhibits IFN signaling pathway through targeting IFNAR1**

He CL *et al*. miR-93-5p promotes HCV-1b infection

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**Abstract**

***AIM***

To investigate the mechanism by which hepatitis C virus (HCV) core protein-induced miR-93-5p regulates the IFN signaling pathway.

***METHODS***

HCV-1b core protein was exogenously expressed in Huh7 cells using pcDNA3.1 (+) vector. The expression of miR-93-5p and interferon receptor 1 (IFNAR1) was measured using quantitative reverse transcription polymerase chain reaction (qRT-PCR) or western blotting. The protein expression and phosphorylation level of STAT1 were evaluated by western blotting. The overexpression and silence of miR-93-5p and IFNAR1 were performed using miR-93-5p’s agomir and antagomir, and pcDNA3.1-IFNAR1 and IFNAR1 siRNA, respectively. IFNAR1 was identified as miR-93-5p’s target using luciferase assay. Cytobiology experiments were also conducted.

***RESULTS***

Serum miR-93-5p expression was increased in patients with HCV-1b infection and decreased to normal level after HCV-1b clearance, but persistently increased in those with pegylated interferon-α resistance, compared with healthy subjects. Serum miR-93-5p expression signature had an AUC value of 0.8359 in distinguishing patients with pegylated interferon-α resistance from those with pegylated interferon-α sensitivity. HCV-1b core protein increased miR-93-5p expression and induced inactivation of the IFN signaling pathway in Huh7 cells. Furthermore, IFNAR1 was identified as a direct target of miR-93-5p, and IFNAR1 restore could rescue miR-93-5p-reduced STAT1 phosphorylation, suggesting that miR-93-5p-IFNAR1 axis regulates the IFN signaling pathway.

***CONCLUSION***

HCV-1b core protein-induced miR-93-5p expression inhibits the IFN signaling pathway through directly targeting IFNAR1, and miR-93-5p-IFNAR1 axis regulates STAT1 phosphorylation. This axis may be a potential therapeutic target for HCV-1b infection.

**Key words:** Hepatitis C virus; miR-93-5p; Interferon receptor 1; the IFN signaling pathway

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**Core tip**: Hepatitis C virus-1b core protein increases miR-93-5p expression and induces inactivation of the IFN signaling pathway. miR-93-5p expression is involved in pegylated interferon-α resistance and directly targets interferon receptor 1 (IFNAR1). miR-93-5p-IFNAR1 axis regulates STAT1 phosphorylation.

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**INTRODUCTION**

Hepatitis C virus (HCV) is a positive-sense single stranded RNA virus that causes hepatitis, jaundice and even fulminant hepatic failure at the beginning infection, but in the majority of persons, the persistent infection of HCV causes cirrhosis and hepatocellular carcinoma (HCC)[1]. It is estimated that chronic hepatitis C impacts approximately 350 million people and constitutes a significant health burden worldwide[2,3]. In the past two decades, interferon has served as the mainstay drug for HCV treatment, and its effect was improved by the addition of ribavirin and then by linking polyethylene glycol to the interferon molecule[4-6]. However, the outcome of IFN-based therapies depends mainly on the patients’ responsiveness and the HCV genotype, especially HCV genotype 1 which has shown no sufficient response to pegylated interferon-α (IFNα)[7]. The core protein is an important component of HCV and plays a crucial role in HCV infection and pegylated IFNα resistance, but the mechanism underlying HCV core protein-induced pegylated IFNα resistance remains unclear.

microRNA (miRNA) is a class of single non-coding RNA with length of ~20 nt, which is involved in the regulation of HCV infection[8]. Several studies indicated that the expression of multiple miRNAs, such as miR-122, miR-1, miR-30, and miR-146a, was regulated by IFN in inhibiting HCV replication[9,10]. Kim *et al*[11] showed that HCV core protein promoted miR-122 destabilization in Huh7 cells, suggesting that miRNA expression was also regulated by HCV core protein. Furthermore, miRNA can also inhibit HCV replication and infectivity, such as let-7 family miRNAs[12], suggesting that miRNA may serve as a target for HCV therapy. The aberrant expression of miRNAs during HCV infection is involved in HCV-associated host pathways[13]. Recent study has shown that miR-93-5p was overexpressed in HCV-associated HCC, and promoted HCC progression[14,15]. However, whether miR-93-5p plays important roles in HCV core protein-associated IFN signaling pathway remains largely unclear.

In the present study, we demonstrated that serum miR-93-5p expression was higher in HCV-1b-infected patients with pegylated IFNα resistance compared with those sensitivity. HCV-1b core protein increased miR-93-5p expression and inhibited the IFN signaling pathway in Huh7 cells. Interferon receptor 1 (IFNAR1) was identified as a direct target of miR-93-5p, and miR-93-5p-INFAR1 axis regulated the IFN signaling pathway.

**MATERIALS AND METHODS**

***Patients and samples***

We enrolled 84 patients who had been identified to be infected with HCV-1b, and enrolled 84 healthy subjects at Southwest Hospital, Third Military Medical University from July 2012 to June 2016. These patients were divided into two groups, one of which was pegylated IFNα resistance and the other was pegylated IFNα sensitivity. The clinical characteristics of individuals was described in Table 1. All samples collection abided by the consents of individuals according to the protocols approved by the Ethics Review Board at Southwest Hospital Institutional Review Board. Serum was isolated from the blood samples within 2 h after collection according to the following steps: 1) 1500 rpm for 10 min, followed by transferring to new tubes; 2) 12000 rpm for 2 min. These steps can prevent contamination by the cellular nucleic acids[16].

***Cell culture***

Human hepatocellular carcinoma cell line, Huh7 was purchased from ATCC, and cultured with DMEM high glucose (Hyclone, United States) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, United States) in a humidified incubator with 5% CO2 at 37 ℃.

***RNA extraction***

Total RNA was isolated from cells and serum using the TRI reagent (Invitrogen, Carlsbad, CA, United States) and the TRIzol LS reagent (Invitrogen), respectively, according to the manufacturer’s instructions. For total RNA isolated from serum, 10 μL of 0.05 μmol/L synthetic *C.elegans* miR-39 (GenePharma, Shanghai, China) was added to each sample after the samples were treated with TRIzol LS reagent. Finally, the total RNA was resuspended in 45 μL of pre-heated (65 ℃) nuclease-free water, and was analyzed using Nanodrop1000 (Thermo, Massachusetts, United States).

***Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)***

Reverse transcription assay was performed using the PrimeScript RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions. One microgramme of total RNA (10 μL) was used for the reverse transcription system, including 4 μL 5 × Reverse transcription buffer, 4 μL nuclease-free H2O, 1 μL Prime RT Enzyme, 1 μL RT Primer. Quantitative PCR assay was conducted using the Permix Ex Taq Kit (TaKaRa) according to the manufacturer’s protocols. Two microlitres of cDNA was used in the qPCR system, containing 10 μL SYBR Premix Ex Taq II, 0.5 μL ROX Reference Dye II, 0.5 μL reverse primer, 0.5 μL forward primer, and 6.5 μL nuclease-free H2O. Running parameters of qPCR: 95 ℃ for 2 min, followed by 40 cycles of 95 ℃ for 15 s and 58 ℃ for 30 s. Melt curves were collected by a range of temperatures from 58 ℃ to 95 ℃, within an increase of 0.5 ℃ per 5 s. Primer sequences were showed in Table 2.

The expression of miR-93-5p and IFNAR1 in Huh7 cells was normalized using the 2-△△*C*t method, RNU6B and β-actinas the references, respectively. Serum miR-93-5p concentration was calculated using a standard curve established by the synthetic miR-93-5p. The standard linearity of miR-93-5p quantification was generated in each qPCR reaction. Experimental qRT-PCR data were normalized using the synthetic *C.elegans* miR-39, described as previous study[16].

***Vector construct***

The DNA oligonucleotides that encode HCV core protein or encode *Homo* *sapiens* IFNAR1 protein were synthesized with flanking *spe* I and *Hind* III restriction enzyme digestion sites. The synthesized DNAs were inserted into pcDNA3.1 (+) vectors (Addgene, United States) using T4 DNA Ligase according to the manufacturer’s instructions. The DNA oligonucleotides containing wild-type or mutant 3’-UTR of IFNAR1 were synthesized with flanking *spe* I and *Hind* III restriction enzyme digestion sites, respectively, and the synthesized DNAs were inserted into psiCHECKTM-2 vectors (Promega, Wisconsin, United States). The sequences of the synthesized DNA were showed in Table 2.

***Oligonucleotide transfection***

The gain- or loss-of-function of miR-93-5p was performed using miR-93-5p agomir (RIBOBIO, Guangzhou, China) or antagomir (RIBOBIO), respectively. Liposome 2000 (Invitrogen) was used for oligonucleotide transfection according to the manufacturer’s instruction.

***Luciferase assay***

HEK293T cells were cultured in 6-well plates and the cells in each well were transfected with 200ng wild-type psiCHECK-IFNAR1-3’UTR or 200ng mutant psiCHECK-IFNAR1-3’UTR, and then miR-93-5p agomir (200nmol/L) or antagomir (200 nmol/L) were transfected into the cells using lipofectamine 2000, respectively. By transfection 24 h, the cells were collected and lysed using luciferase reporter assay system according to the manufacturer’s instructions, followed by fluorescence activity being detected using GloMax 20/20 Luminometer.

***Western blotting***

Total protein from tissues and cells was extracted using the RIPA Lysis and Extraction Buffer (Thermo) according to the manufacturer’s instructions. Western blotting assay was performed according to the standard protocol. Briefly, total proteins were separated using eight or ten percent SDS-PAGE gels, which were followed by transferred on PVDF membranes. Five percent skim milk or three percent BSA solution (used for p-STAT1 antibody) was used to block the PVDF membranes. Then the anti-IFNAR1 Abs (Abcam, Cambridge, UK), anti-STAT1 Abs (Cell Signaling Technology, Boston, United States), anti-p-STAT1 Abs (Cell Signaling Technology) and anti-GAPDH Abs (Cell Signaling Technology) were used to incubate the PVDF membranes for overnight, which had been cut based on the molecular weight. Then HRP-conjugated secondary Abs (Zhongshan Biotechnology, Beijing, China) was used to incubate the PVDF membranes for 1 h. After the PVDF membranes were washed using 1 × TBST solution three times, they were visualized using the SuperSignal West Dura Extended Duration Substrate kit (Thermo).

***Statistical analysis***

All data were presented as mean ± SD. The difference between two groups was analyzed using the Mann-Whitney test or the Two-tailed Student’s *t*-test. The one-way ANOVA was used for three or more groups. The relationship between the expression of miR-93-5p and IFNAR1 mRNA was evaluated using the Pearson’s correlation. A *P*-value of < 0.05 was considered statistically significant. All statistical analyses and the generation of graphs were performed using GraphPad Prism 6.0 (Graphpad Software Inc, California).

**RESULTS**

***Serum miR-93-5p expression is increased in*** hepatitis C virus***-1b-infected patients***

miR-93-5p has been shown overexpressed in HCV-infected HCC tissues[14]. To determine whether miR-93-5p expression was also increased in serum of HCV-infected patients, we collected 168 serum samples, 84 samples from 84 patients with HCV-1b infection before pegylated IFNα treatment, and the other 84 serum samples from the same patients after pegylated IFNα treatment by 24 wk. PCR finally confirmed 34 patients with pegylated IFNα resistance and 50 patients with pegylated IFNα sensitivity.

To measure serum miR-93-5p concentration, we established a dynamic range of miR-93-5p quantification. The synthetic single-strand miR-93-5p was serially diluted by 10-fold from concentrations of 1 to 0.00001 fmol, and qRT-PCR experiments showed the linearity of miR-93-5p quantification (Figure 1A). For the samples before pegylated IFNα treatment, serum miR-93-5p expression was significantly increased in HCV-1b-infected patients compared with healthy subjects (*P* < 0.0001) (Figure 1B), and it was also increased in the patients with pegylated IFNα sensitivity compared with healthy subjects (*P* = 0.0100), and interestingly it was higher in the patients with pegylated IFNα resistance than that in those sensitivity (*P* < 0.0001) (Figure 1C). For the samples after pegylated IFNα treatment, serum miR-93-5p expression was persistently increased in the patients with pegylated IFNα resistance (*P* < 0.0001) (Figure 1D), but it was decreased in the patients with pegylated IFNα sensitivity, similar to that in healthy subjects (Figure 1E). These data suggest that high level of miR-93-5p in serum is involved in HCV-1b infection and pegylated IFNα resistance.

***miR-93-5p concentration in serum of patients with*** hepatitis C virus***-1b infection is involved in pegylated IFNα resistance***

To determine whether serum miR-93-5p concentration can serve as a biomarker for predicting pegylated IFNα resistance, receiver operating characteristics (ROC) curve analysis was performed. The results showed an AUC value of 0.8846 for serum miR-93-5p in distinguishing HCV-1b-infected patients from healthy subjects (Figure 2A), an AUC value of 0.8562 in differing sensitivity from healthy (Figure 2B), an AUC value of 0.9265 in distinguishing resistance from healthy (Figure 2C), and an AUC value of 0.8359 in differing resistance from sensitivity (Figure 2D). The detailed information is showed in Table 3. These data suggest that serum miR-93-5p concentration may serve as a biomarker for HCV-1b-infected patients with pegylated IFNα resistance.

hepatitis C virus***-1b core protein increases miR-93-5p expression and induces inactivation of the IFN signaling pathway***

HCV core protein plays an important role in HCV infection. To determine whether HCV-1b infection could regulate miR-93-5p expression, HCV-1b core protein was enforcedly expressed in Huh7 cells (*P* = 0.0032) (Figure 3A). This approach was widely used in HCV studies due to its capability in supporting HCV replication. Previous study has shown that HCV-1b core protein decreased mature miR-122 expression[11]. Thus, we repeated these experiments and confirmed that HCV-1b core protein could reduce miR-122 expression (*P* = 0.0207) (Figure 3B), suggesting that exogenous HCV-1b core protein worked in Huh7 cells. Then, we found that HCV-1b core protein significantly increased miR-93-5p expression in Huh7 cells (*P* = 0.0004) (Figure 3C).

Although the treatment of pegylated interferon plus ribavirin was used, the sustained virological response (SVR) rate is around 50-79% for HCV type 1/4 and 75-94% for HCV type 2/3[17], suggesting that IFN-α resistance largely existed in HCV-1b infection. Thus, we speculated whether HCV-1b core protein could regulate the IFN signaling pathway. We initially measured the protein expression of IFNAR1 and STAT1, as well as the phosphorylation level of STAT1. As expected, our finding showed that HCV-1b core protein significantly decreased the protein expression of IFNAR1 and the phosphorylation level of STAT1, but the protein expression of STAT1 was not changed (Figure 3D and E). These data suggesting that HCV-1b core protein induced inactivation of the IFN signaling pathway.

***Interferon receptor 1 is a direct target of miR-93-5p***

Next, we need to determine whether miR-93-5p is involved in HCV-1b core protein-induced inactivation of the IFN signaling pathway. As well known that miRNA exerting its function depends on its target. Thus, we used five databases (TargetScan, PicTar, RNA22，PITA, and MiRanda) to predict the targets of miR-93-5p, and found that IFNAR1 may be a potential target of miR-93-5p. IFNAR1 mRNA had three binding sites of miR-93-5p in the 3’UTR region. To confirm whether IFNAR1 is a target of miR-93-5p, we constructed the vectors containing wild-type 3’-UTR or mutant 3’UTR of IFNAR1 mRNA using psiCHECK vector (Figure 4A). Luciferase assay revealed that miR-93-5p agomir significantly reduced the relative luciferase activity at binding site 1 (P < 0.0001) or site 2 (P = 0.0173), rather than site 3. However, at the mutant binding sites the relative luciferase activity had no significant change, compared with NC (Figure 4B). Furthermore, miR-93-5p agomir also decreased the protein (P = 0.0261) and mRNA (P = 0.0065) expression of IFNAR1 in Huh7 cells compared with NC, while opposite results were found after miR-93-5p antagomir treatment (P = 0.0109, P = 0.0089) (Figure 4C, D and E). Together, these data suggested that IFNAR1 is a direct target of miR-93-5p.

***miR-93-5p-IFNAR1 axis regulates the IFN signaling pathway***

STAT1, a transcription factor, plays a crucial role in the IFN signaling pathway. Several studies have shown that IFNAR1 regulates the phosphorylation level of STAT1[18-20]. However, it is unclear whether miR-93-5p-IFNAR1 axis could regulate the phosphorylation level of STAT1 in Huh7 cells. Thus, we conducted the enforced expression of IFNAR1 and its silence in Huh7 cells using pcDNA3.1 (+) vectors and IFNAR1 siRNA, respectively (Figure 5A). Western blotting experiments showed that IFNAR1 knockdown significantly decreased the phosphorylation level of STAT1 (*P* = 0.0021), but not the protein expression of STAT1, whereas IFNAR1 overexpression significantly increased the phosphorylation level of STAT1 (*P* = 0.0153) (Figure 5B and C). Furthermore, miR-93-5p agomir significantly decreased the phosphorylation level of STAT1 (*P* = 0.0077), whereas miR-93-5p inhibitors significantly increased it (*P* = 0.0262) (Figure 5D and E). To further confirm that miR-93-5p-IFNAR1 axis could regulate STAT1 phosphorylation, a rescue assay was carried out. The results showed that IFNAR1 overexpression rescued miR-93-5p-induced the decrease of STAT1 phosphorylation level in Huh7 cells (Figure 5F). These data suggested that miR-93-5p-IFNAR1 axis regulated the STAT1 signaling pathway.

**DISCUSSION**

In the present study, we found that serum miR-93-5p expression was increased in patients with HCV-1b infection compared with healthy subjects, and was higher in HCV-1b-infected patients with pegylated IFNα resistance than those sensitivity. We also found that HCV-1b core protein could increase miR-93-5p expression and decrease IFNAR1 expression, and further identified that IFNAR1 is a direct target of miR-93-5p. Furthermore, our findings showed that miR-93-5p-IFNAR1 axis could regulate STAT1 phosphorylation.

miR-93-5p belongs to the miR-106-25 cluster which plays a crucial role in cancer development. Yen *et al*[14] found that three miRNAs in the miR-106-25 cluster were overexpressed in HCC tissues and their data suggested that miR-93-5p was upregulated in HCV-associated HCC tissues. In addition, miR-93-5p overexpression was also involved in HCV-positive cirrhosis[21]. In our study, we found that miR-93-5p overexpression was associated with pegylated IFNα resistance to HCV-1b, which is the most prevalent type of HCV in China and highly resists to IFNα treatment. HCV core protein, an important component of HCV, promoted the dysregulation of miRNAs, such as miR-122[11]. However, whether HCV core protein regulates miR-93-5p expression remains largely unclear. Our findings demonstrated that HCV core protein increased miR-93-5p expression in Huh7 cells.

Ohta *et al*[15] demonstrated that miR-93 directly targeted PTEN and CDKN1A, thereby activating proliferation and inhibiting apoptosis through the c-Met/PI3K/Akt pathway. In our study, we integrated five databases to find that IFNAR1, a receptor of IFNα and IFNβ, was a potential target of miR-93-5p and further confirmed it, implying that miR-93-5p had multiple targets in hepatocyte, but our data suggested that miR-93-5p-IFNAR1 axis might be mainly involved in HCV-1b infection. Recently, Ma *et al*[22] showed that miR-93-5p/IFNAR1 axis promotes gastric cancer metastasis through activating the STAT3 signaling pathway. These data strongly support our finding that IFNAR1 is a target of miR-93-5p in hepatocyte cells. We believe that similar mechanism in different types of cells may play different roles. As well known that IFNAR1 plays a crucial role in the IFN signaling pathway. Our study then confirmed that miR-93-5p-IFNAR1 axis regulates the phosphorylation level of STAT1, which is an important transcription factor in the IFN signaling pathway. Zhao *et al*[23] have confirmed that IFNAR mediates IFN-α-triggered STAT signaling pathway, and inhibition of this pathway could increase HCV RNA replication. These data strongly support our finding that miR-93-5p-IFNAR1 axis regulated STAT1 phosphorylation, thereby affecting the therapeutic effect of pegylated IFNα.

In addition, HCV infection could reduce the protein expression of STAT1 and STAT3 by ubiquitination approach[24], but our data revealed that HCV-1b infection could regulate STAT1 phosphorylation by miR-93-5p-IFNAR1 axis. Although we identified the role and mechanism of miR-93-5p in regulating the IFN signaling pathway in Huh7 cells, an *in vivo* study needs to be performed in the future.

In conclusion, we found that HCV-1b core protein increased miR-93-5p expression and identified that IFNAR1 is a direct target of miR-93-5p. Moreover, miR-93-5p-IFNAR1 axis regulated STAT1 phosphorylation, suggesting this axis might be a novel therapeutic target for HCV-1b infection (Figure6).

**ARTICLE HIGHLIGHTS**

***Research background***

hepatitis C virus (HCV)-1b core protein is an important component of HCV and plays a crucial role in HCV infection and pegylated IFNα resistance, but the mechanism underlying HCV core protein-induced pegylated IFNα resistance remains unclear.

***Research motivation***

Our findings highlight the mechanism that HCV-1b core protein induces pegylated IFNα resistance *via* regulating miR-93-5p-interferon receptor 1 (IFNAR1) axis. This axis may be a potential therapeutic target for HCV-1b treatment.

***Research objectives***

The objective of this study is to elucidate why HCV-1b causes pegylated IFNα resistance. It is partially realized and provides a clue for drug design of HCV-1b treatment.

***Research methods***

The research methods included cell culture, RNA extraction, qRT-PCR, vector construct, oligonucleotide transfection, luciferase assay, and western blotting. Data analysis was performed using multiple statistical methods that include the Mann-Whitney test, the Two-tailed Student’s *t*-test, the one-way ANOVA, and the Pearson’s correlation, and using software, GraphPad and Image-pro plus. Eighty-four patients with HCV-1b infection and 84 healthy subjects were enrolled in this study.

***Research results***

This study found that serum miR-93-5p expression was increased in patients with HCV-1b infection and HCV-1b core protein increased miR-93-5p expression, and identified that IFNAR1 is a target of miR-93-5p, and further demonstrated that miR-93-5p-IFNAR1 axis regulated the IFN signaling pathway.

The research findings, their contributions to the research in this field, and the problems that remain to be solved should be described in detail.

***Research conclusions***

This study found that HCV-1b increases miR-93-5p expression, IFNAR1 is a target of miR-93-5p in hepatocyte, and miR-93-5p-IFNAR1 axis regulates the IFN signaling pathway. This study also provided some evidence that HCV-1b induces pegylated IFNα resistance by regulating miR-93-5p-IFNAR1 axis, suggesting that miR-93-5p-IFNAR1 axis might be a potential therapeutic target of HCV-1b infection.

***Research perspectives***

This study demonstrated the mechanism by which miR-93-5p inhibited the IFN signaling pathway *in vitro*, but *in vivo* study needs to be performed in the future. The design of siRNAs which exclusively destroy the miR-93-5p-IFNAR1 axis may be important for improving the therapeutic effect of pegylated IFNα.

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Grade D (Fair): 0

Grade E (Poor): 0

**Table 1 Characteristics of patients with hepatitis C virus-1b infection**

|  |  |  |  |
| --- | --- | --- | --- |
| **Variable** | **High expression of miR-93-5p** | **Low expression of miR-93-5p** | ***P*-value** |
| Gender |  |  | 0.382 |
| Male | 25 | 20 |  |
| Female | 17 | 22 |  |
| Age |  |  | 0.189 |
| Median (range) |  |  |  |
| ＞44 | 19 | 26 |  |
| ≤ 44 | 23 | 16 |  |
| HCV-1b treatment |  |  | 0.001 |
| Resistance | 25 | 9 |  |
| Sensitivity | 17 | 33 |  |

Differences between variable were analyzed by the *χ*2 test. HCV: Hepatitis C virus.

**Table 2 Primers sequences**

|  |  |
| --- | --- |
| **mRNA** | **Sequence** |
| β-actin forward | 5’-TGTCCACCTTCCAGCAGATGT-3’ |
| β-actin reverse | 5’-TGTCACCTTCACCGTTCCAGTT-3’ |
| IFNAR1 forward | 5’-TGGTGACAGCGTGAGACTCTT-3’ |
| IFNAR1 reverse | 5’-GCAGTAGCCAGCAGCATCAG-3’ |
| miR-93-5p forward | 5′- GCCGCCAAAGTGCTGTTC-3′ |
| miR-93-5p reverse | 5′-CAGAGCAGGGTCCGAGGTA-3′ |
| U6 forward | 5′-CAGCACATATACTAAAATTGGAACG-3′ |
| U6 reverse | 5′-ACGAATTTGCGTGTCATCC-3′ |

IFNAR1: Interferon receptor 1.

**Table 3 Receiver operating characteristics curve analyses of serum miR-93-5p concentration**

|  |  |  |  |
| --- | --- | --- | --- |
| **AUC** | **Sensitivity** | **Specificity** | **Cut-off value** |
| 0.8846 (Infection *vs* Healthy) | 76.19% | 100.00% | 0.009774 amol/μL |
| 0.8562 (Sensitivity *vs* Healthy) | 70.00% | 100.00% | 0.009774 amol/μL |
| 0.9265 (Infection *vs* Resistance) | 85.29% | 100.00% | 0.010870 amol/μL |
| 0.8359 (Resistance *vs* Sensitivity) | 76.47% | 100.00% | 0.030300 amol/μL |

Infection: HCV-1b-infected patients; Healthy: Healthy subjection; Sensitivity: Pegylated IFNα sensitivity; Resistance: Pegylated; IFNα resistance.

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**Figure 1 Serum miR-93-5p expression is increased in hepatitis C virus-1b-infected patients.** A: The linearity of miR-93-5p quantification; B: Box shows miR-93-5p concentration in serum of 84 patients with HCV-1b infection, compared with that of 84 healthy subjects; C: Box shows miR-93-5p concentration in serum of 50 HCV-1b-infected patients with pegylated IFNα sensitivity and 34 HCV-1b-infected patients with pegylated IFNα resistance, compared with that of 84 healthy subjects; D: Scatter shows serum miR-93-5p concentration in 84 healthy subjects, 50 HCV 1b-infected patients with pegylated IFNα sensitivity before or after treatment; E: Scatter shows serum miR-93-5p concentration in 84 healthy subjects, 34 HCV 1b-infected patients with pegylated IFNα resistance before or after treatment. HCV: Hepatitis C virus; IFNα: Interferon-α.

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**Figure 2 miR-93-5p concentration in serum of patients with hepatitis C virus-1b infection is involved in pegylated interferon-α resistance.** A: Receiver operating characteristics (ROC) curve shows an AUC value of 0.8846 for serum miR-93-5p concentration in distinguishing HCV-1b-infected patients from healthy subjects; B and C: ROC curve shows AUC values of 0.8562 and 0.9265 in distinguishing HCV-1b-infected patients with pegylated IFNα sensitivity and resistance from healthy subjects, respectively; D:ROC curve shows an AUC value of 0.8359 in distinguishing HCV-1b-infected patients with pegylated IFNα resistance from those with pegylated IFNα sensitivity. HCV: Hepatitis C virus; IFNα: Interferon-α.

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**Figure 3 hepatitis C virus -1b core protein increases miR-93-5p expression and inactivated the interferon signaling pathway.** A: HCV core protein is enforcedly expressed in Huh7 cells using pcDNA3.1 (+) vector. Western blot shows HCV core protein expression. These results were repeated two times. B: Bar shows miR-122 expression in Huh7 cells transfected with pcDNA 3.1 (+) empty vector or HCV core-pcDNA3.1 (+) vector. U6 as internal reference. These results were repeated three times. C: Bar shows miR-93-5p expression in Huh7 cells transfected with pcDNA 3.1 (+) empty vector or HCV core-pcDNA3.1 (+) vector. U6 as internal reference. These results were repeated three times. D and E: Western blot shows the protein expression of IFNAR1 and STAT1, as well as the phosphorylation level of STAT1 in Huh7 cells which were transfected with pcDNA 3.1 (+) empty vector or HCV core-pcDNA3.1 (+) vector. These results were repeated two times. HCV: Hepatitis C virus, IFNAR1: Interferon receptor 1.

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**Figure 4 FNAR1 is a direct target of miR-93-5p.** A: The binding sites of miR-93-5p on the IFNAR1 3’UTR region, and the design of mutant binding sites on the IFNAR1 3’UTR for miR-93-5p; B: Luciferase assay shows that miR-93-5p agomir inhibits the relative luciferase activity in the binding site 1 or 2-expressing HEK293T cells, but not in the binding site 3- or mutant binding sites-expressing HEK293T cells. These results were repeated six times; C and D**:** Western blot shows the protein expression of IFNAR1 in Huh7 cells transfected with ago-NC (200 nmol/L), miR-93-5p agomir (200 nmol/L), anta-NC (200 nmol/L), and miR-93-5p antagomir (200 nmol/L). These results were repeated two times; E: qRT-PCR shows the mRNA expression of IFNAR1 in Huh7 cells transfected with ago-NC (200 nmol/L), miR-93-5p agomir (200 nmol/L), anta-NC (200 nmol/L), and miR-93-5p antagomir (200 nmol/L). β-actin as internal reference. These results were repeated three times. IFNAR1: Interferon receptor 1.

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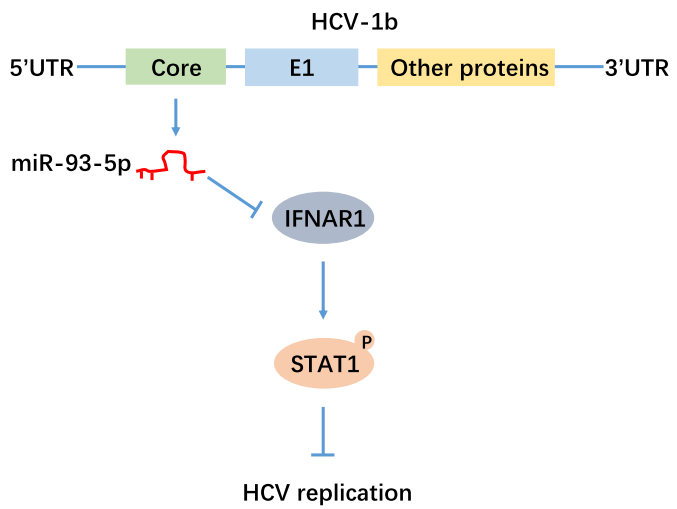
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**Figure 5 miR-93-5p-IFNAR1 axis regulates the interferon signaling pathway.** A: Western blot shows the protein expression of IFNAR1 in Huh7 cells transfected with NC (200 ng/mL), siRNA (200 ng/mL), vector (200 ng/mL), or IFNAR1 (200 ng/mL). These results were repeated two times. B and C: Western blot shows the phosphorylation level of STAT1 and the protein expression of STAT1 in Huh7 cells transfected with NC (200 ng/mL), siRNA (200 ng/mL), vector (200 ng/mL), or IFNAR1 (200 ng/mL). These results were repeated two times. D and E: Western blot shows the phosphorylation level of STAT1 and the protein expression of STAT1 in Huh7 cells transfected with ago-NC (200 nmol/L), miR-93-5p agomir (200 nmol/L), antago-NC (200 nmol/L), or miR-93-5p antagomir (200 nmol/L). These results were repeated two times; F and G: Western blot shows the phosphorylation level of STAT1 and the protein expression of STAT1 and IFNAR1 in Huh7 cells transfected with ago-NC (200 nmol/L), miR-93-5p agomir (200 nmol/L), or co-transfected with miR-93-5p agomir (200 nmol/L) and IFNAR1 (200 ng/mL). IFNAR1: Interferon receptor 1.



**Figure 6 Schematic figure on the mechanism that hepatitis C virus-1b core protein induces inactivation of the interferon signaling pathway *via* regulating miR-93-5p.**