MiR-221 accentuates IFN's anti-HCV effect by downregulating SOCS1 and SOCS3

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MiR-221 was reported to be upregulated and play roles in tumorigenesis of hepatitis C virus (HCV) associated hepatocellular carcinoma (HCC). However, the role of miR-221 in HCV infection remains unknown. In this study, it was found that miR-221 was upregulated in serum of HCV chronic hepatitis patients and Huh7.5.1 cells infected with HCVcc. Further studies indicated that miR-221 mimic could accentuate anti-HCV effect of IFN-α in HCVcc model, miR-221 mimic could further repressed 10% HCV RNA expression and 35–42% HCV core or NS5A protein expression in HCVcc infected Huh7.5.1 cells treated with 100 IU/ml IFN-α, and miR-221 inhibitor resulted in the reverse effects. Furthermore, two members of suppressor of cytokine signaling (SOCS) family, SOCS1 and SOCS3, which are well established inhibitory factors on IFN/JAK/STAT pathway, were identified as the targets of miR-221 and were involved in the effect of miR-221. In conclusion, miR-221 could accentuate IFN's anti-HCV effect by targeting SOCS1 and SOCS3.

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Introduction

Hepatitis C virus (HCV) is a blood-transmitted human virus and it is a global health problem with estimated of 170 million infected individuals worldwide (Chisari, 2005). Seventy percent of acute infections are rapidly established as chronic infections, which is a leading cause of liver cirrhosis and hepatocellular carcinoma (HCC) (Bowen and Walker, 2005).

In the past decades, studies have focused on investigating the genes and proteins underlying HCV infection, the host defenses and the progression of HCC (Gale and Foy, 2005; Garaigorta and Chisari, 2010; Liu and Gale, 2010). Recently, an increasing number of reports described a new class of small regulatory RNA molecules termed microRNAs (miRNAs) that are implicated in the progression of hepatitis virus associated HCC (Negrini et al., 2011; Zhang et al., 2010; Braconi et al., 2010; Diaz et al., 2013) and in response to virus infection (Berkhout and Jeang, 2007; Watanabe et al., 2007; Marquez et al., 2010). Specially, miR-122 has been shown to intensify the replication of HCV by targeting the viral 5′ non-coding region (Jopling et al., 2005). Moreover, the locked nucleic acid (LNA)–modified oligonucleotide (SPC3649) complementary to miR-122 leads to long-lasting suppression of HCV viremia, with no evidence of viral resistance or side effects in HCV chronically infected chimpanzees (Lanford et al., 2010). The use of an antisense oligonucleotide that sequesters mature miR-122 in a highly stable heteroduplex, miravirsen, in patients with chronic HCV genotype 1 infection showed prolonged dose-dependent reductions in HCV RNA levels without evidence of viral resistance (Jansen et al., 2013). These findings suggest miRNAs are involved in liver tumorigenesis at early stage of HCV infection and have the potential to become novel drug targets in virally induced infectious or malignant diseases.

MiR-221 has been reported in a variety of cancers including HCC (Garofalo et al., 2012). MiR-221 is involved in liver fibrosis and HCC angiogenesis and tumorigenesis by regulating its target proteins (Park et al., 2011; Gramantieri et al., 2009; Pineau et al., 2010; Fornari et al., 2008; Ogawa et al., 2012; Santhekadur et al., 2012; Callegari et al., 2012). MiR-221 regulates CDKN1C/p57 and CDKN1B/p27 expressions (Park et al., 2011); miR-221 targets Bmf and correlates with tumor multifocality (Gramantieri et al., 2009); overexpression of miR-221 contributes to tumorigenesis (Pineau et al., 2010); the knockdown of miR-221 blocks hepatocellular carcinoma and promotes survival (Fornari et al., 2008); miR-221 activates hepatic stellate cells and contributes to liver fibrosis (Ogawa et al., 2012); miR-221 is involved in the promotion of tumor angiogenesis by staphylococcal nuclease domain containing 1 (SND1) (Santhekadur et al., 2012); miR-221 is found to promote liver tumorigenicity in transgenic mouse model (Callegari et al., 2012). Although miR-221 has been reported to play
important roles in HCV associated HCC tumorigenesis, the mechanistic details of direct role of miR-221 in the regulation of HCV replication however remain unclear. In the present study, miR-221 was found to accentuate IFN's effect of repressing HCV replication by targeting suppressor of cytokine signaling 1 (SOCS1) and suppressor of cytokine signaling 3 (SOCS3).

Results

MiR-221 is upregulated by HCV infection

In serum of HCV infected patients with chronic hepatitis, miR-221 was upregulated around 4.5 folds in serum of patients with CHC ($n=34$) compared with healthy blood donors ($n=22$) (Fig. 1A). To verify whether the upregulation of miR-221 is associated with HCV infection, the relative expression of miR-221 in HCVcc infected Huh7.5.1 cells was evaluated. Seventy two hours after HCVcc infection, miR-221 was upregulated around 5.3 folds in Huh7.5.1 cells (Fig. 1B). Huh7.5.1 cells infected with HCVcc were harvested at different time points, and the relative expressions of HCV RNA and miR-221 were determined by real-time PCR. GAPDH and U6 are used as an internal control. Column, mean of three independent experiments; bars, SD; *, $P<0.01$, **, $P<0.001$. 

![Fig. 1](image-url)

Fig. 1. MiR-221 is upregulated by HCV infection. (A) The expression of miR-221 in serum of HCV chronic hepatitis patients. (B) The expression of miR-221 in Huh7.5.1 cells 72 h after HCVcc infection. (C) Expressions of HCV RNA and miR-221 in Huh7.5.1 cells at the indicated time points post-infection. (D) Expressions of HCV RNA and miR-221 in Huh7.5.1 cells after infection with different levels of HCVcc. Intracellular HCV RNA and miR-221 are analyzed by RT-PCR. GAPDH and U6 are used as an internal control. Column, mean of three independent experiments; bars, SD; *, $P<0.01$, **, $P<0.001$. 

Intracellular HCV RNA increased rapidly and reached to a relative plateau at day 3 post-infection, and miR-221 expression increased to a relative stable level at day 3 post-infection (Fig. 1C). In contrast, there were no significant changes in expressions of miR-221 in uninfected group throughout the experiment (data not shown). In addition, Huh7.5.1 cells were infected with different
amounts of HCV for 3 days. The expression of HCV RNA and miR-122 in each group (10^5 IU/ml group, 10^6 IU/ml group and 10^7 IU/ml group) increased to different levels (Fig. 1D). These results indicated that HCV infection could induce the upregulation of miR-221.

MiR-221 accentuates IFNα's repression of HCV replication

To investigate the possible role of miR-221 in HCV infection, the effect of miR-221 on HCV replication was evaluated in Huh7.5.1 cells infected with HCVcc and transfected with or without, miR-221 mimic, miR-221 inhibitor, mm-NC or mi-NC. The expression of miR-221 was increased 23.6 folds in cells transfected with 20 nM miR-221 mimic, but decreased 68.4% in cells transfected with 20 nM miR-221 inhibitor (Fig. 2A). By RT-PCR assay, 100 IU/ml IFNα could repress more than 80% HCV replication in HCVcc infected Huh7.5.1 cells. In addition, HCV RNA expression was further deceased by 43.6% by miR-221 mimic, but increased 36.5% by miR-221 inhibitor (Fig. 2B). However, miR-221 mimic or inhibitor showed no effect on HCV RNA expression in HCVcc infected Huh7.5.1 cells without IFNα treatment (Fig. 2B). Similar results were seen by Western blotting. The expressions of HCV core and NS5A protein were further decreased 35% and 42%, respectively, in miR-221 mimic transfected cells; but increased 105% and 123%, respectively, in miR-221 inhibitor transfected cells only with IFNα treatment (Fig. 2B). These results indicate that miR-221 is most likely involved in the repression effect of IFNα signaling on HCV replication.

A number of proteins, including IRF7, ISG15, OAS1 and PKR induced by the JAK/STAT pathway play a role in the antiviral responses of IFNα. We directly tested whether miR-221 mimic and miR-221 inhibitor could affect the expression of these ISGs. In IFN-α treated Huh7.5.1 cells, all of these 4 ISGs mRNA expressions were significantly increased by RT-qPCR, miR-221 mimic could further increase ISG15 and IRF7, but miR-21 inhibitor decreased their mRNA levels, to 45.6-fold and 18.7-fold and 28.7-fold and 16.2-fold, respectively (Fig. 3B). Taken together, these findings indicate that miR-221 increases the expression of several important anti-HCV factors including ISG15 and IRF7 which correlates with decreased HCV replication.

SOCS1 and SOCS3 are the targets of miR-221

It is generally accepted that miRNAs exert their function through regulating the expression of their downstream target genes. SOCS1 and SOCS3 were predicted as potential targets of miR-221 by TargetScan and PicTar. The 3'-UTR of SOCS1 and SOCS3 mRNA contained a complementary site for the seed region of miR-221 (Fig. 4A). To validate whether SOCS1 and SOCS3 are the targets of miR-221, 3'UTR fragment of SOCS1 and SOCS3 containing miR-221 binding sequence were subcloned downstream of a luciferase reporter vector. In HEK293T cells cotransfected with the reporter
plasmids and miR-221 mimic, mutant miR-221 or mm-NC, the luciferase activity of the reporter that contained SOCS1 and SOCS3 3'-UTR was significantly suppressed by miR-221 mimic, but the luciferase activity of the reporter that contained SOCS1 and SOCS3 mutant 3'-UTR remained no significant difference (Fig. 4B). Using mutant miR-221, the luciferase activity of the reporter that contained mutant SOCS1 and SOCS3 3'-UTR was significantly suppressed (Fig. 4B). The results indicated that miR-221 may suppress gene expression through miR-221 binding sequence at the 3'-UTR of SOCS1 and SOCS3. Furthermore, transfection of miR-221 mimic decreased SOCS1 and SOCS3 expressions and transfection of miR-221 inhibitor increased SOCS1 and SOCS3 expressions in Huh7.5.1 cells at both mRNA (by qRT-PCR) and protein level (Western blotting) (Fig. 4C and D), suggesting that SOCS1 and SOCS3 expressions could be inhibited by miR-221. Together, these results showed that miR-221 could regulate the expressions of human SOCS1 and SOCS3 mRNA.

**Fig. 3.** MiR-221 upregulates IFN-α induced JAK/STAT pathway. (A) MiR-221 rescued JAK/STAT signaling pathway. The key molecular of JAK/STAT pathway including p-JAK1, p-STAT1 and p-STAT3 are deregulated in IFN-α treated Huh7.5.1 cells transfected with miR-221 mimic or miR-221 inhibitor. (B) ISGs including ISG15 and IRF7 are further upregulated by miR-221 in IFN-α treated Huh7.5.1 cells. GAPDH is used as an internal control. Column, mean of three independent experiments; bars, SD; *P < 0.01.

MiR-221 is involved in IFN's repression on HCV replication by targeting SOCS1 and SOCS3

To identify whether inhibition of SOCS1 and SOCS3, just like miR-221 restoration, also resulted in HCV repression, the effects of knockdown of SOCS1 and SOCS3 on HCV replication were examined. Seventy two hours after transfection with SOCS1, SOCS3 specific siRNAs or NC siRNA, the expressions of SOCS1 and SOCS3 were decreased at 48.3% and 81.3% respectively (Fig. 5A). The knockdown of SOCS1 or SOCS3 upregulated the phosphorylation of JAK1, STAT1 and STAT3 (Fig. 5A). Furthermore, knockdown of both SOCS1 and SOCS3 enhanced the repression of HCV replication by IFN-α (Fig. 5B), and miR-221 showed no further effect in such SOCS1 or SOCS3 knockdown cells (Fig. 5B). These results indicate that SOCS1 and SOCS3 are most likely involved in the effect of miR-221 on IFN-α signaling.

Together with the above findings, it was concluded that miR-221 rescue JAK/STAT signaling by targeting SOCS1 and SOCS3, and rescued IFN/JAK/STAT signaling repression HCV replication more efficiently, thus miR-221 accentuates anti-HCV activity of IFN-α by targeting SOCS1 and SOCS3 (Fig. 5C).

Discussion

Altered miRNA expression during viral infections includes both miRNAs that target viral sequences as well as miRNAs that target host genes that influence the course of viral replication and pathogenesis (Kumar, 2011). A number of miRNAs have been reported to be involved in HCV infection. MiR-491 was involved in regulation of HCV replication via the PI3 kinase/Akt pathway (Ishida et al., 2011); miR-155 was found to be associated with HCV infection (Bala et al., 2012); and let-7b can also regulate HCV replication (Cheng et al., 2012).

In the present study, we demonstrated miR-221 could help to repress HCV replication, which might provide new insights into the mechanisms underlying HCV infection, but also raise a doubt on the exact functions of miR-221. As reported by previous studies, miR-221 is regarded as an oncogene which could enhance tumorigenesis (Janssen et al., 2013; Garofalo et al., 2012; Park et al., 2011; Gramantieri et al., 2009; Pineau et al., 2010; Fornari et al., 2008; Ogawa et al., 2012). Our findings suggest that miRNAs might play dual roles in HCV infection. Research on miR-122 support such a hypothesis. On the one hand, miR-122 enhances the replication, translation and infectious virus production of HCV (Chang et al., 2008; Jangra et al., 2010; Roberts et al., 2011); overexpression of miR-122 even supports the entire HCV life cycle in HepG2 cells (Narbus et al., 2011). On the other hand, miR-122 plays an
inhibitory effect on HCC tumorigenesis since it was found that miR-122 suppresses cell proliferation and induces cell apoptosis in HCC by targeting Wnt/β-catenin pathway (Xu et al., 2012), inhibiting tumorigenicity (Bai et al., 2009; Hsu et al., 2012). Moreover, miR-122 was reported to repress HBV transcription in a p53-dependent manner (Wang et al., 2012; Fan et al., 2011), which could suggest a possible virus type dependent role of miRNAs. As Sendi (2012) discussed, miR-122 might play the dual role of in viral hepatitis, and therapeutic applications of miR-122 might differ based on the underlying disease. Thus, miRNAs could play different roles in viral infection and virus-associated tumorigenesis.

As the IFN system represents an integral part of the mammalian innate immunity, it is likely that miRNAs might either target pattern recognition receptors and signaling components involved in the IFN induction after pathogen recognition, or alter expression of proteins involved in the IFN response such as the JAK/STAT pathway. Several cellular miRNAs that are induced during viral infection were reported to negatively modulate the IFN pathway. MiR-26a, miR-34a, miR-145, and let-7b were proved to be IFN-β inducible and directly regulated IFN-β expression during infection as a negative feedback loop (Witwer et al., 2010). MiR-146 limits interleukin-1 receptor associated kinase 1 (IRAK1) and TNF receptor associated factor 6 (TRAF6) expression and directly attenuated transcriptional induction via the IFN-α/β receptor by limiting expression of STAT1 (Taganov et al., 2006; Tang et al., 2009). Inducible expression of miR-155 was observed during both bacterial and viral infections, as well as after exposure of cells to proinflammatory cytokines such as IFN-β, IFN-γ, or tumor necrosis factor alpha (TNF-α) (O’Connell et al., 2007).

It is generally accepted that miRNAs exert their function through regulating the expression of their target genes. No candidate related to IFN/JAK/STAT pathway was predicted as the target of miR-221 by TargetScan and PicTar. Interesting, SOCS1 and SOCS3 were predicted as the targets of miR-221. SOCS family is a mainly negative regulator of JAK/STAT signaling. So far, 8 members of the SOCS family have been identified, termed SOCS1 to SOCS-7 and CIS (Yoshimura, 2009). Both SOCS1 and SOCS3 can inhibit JAK tyrosine kinase activity directly through their kinase inhibitory regions (KIR) (Yasukawa et al., 1999). For HCV infection, SOCS1 suppression was associated with abnormal B cell activation (Moorman et al., 2009); HCV core protein exerts an inhibitory effect on SOCS1 expression (Miyoshi et al., 2005); and increased expression of SOCS3 was associated with non-response to antiviral therapy (Walsh et al., 2006). Furthermore, SOCS1 has been reported to be a target of miR-155 and miR-221 (Cardoso et al., 2012; Lu et al., 2011), while SOCS3 is the target of miR-203 in gingival epithelial cells (Moffatt and Lamont, 2011). We identified SOCS1 and SOCS3 as the targets of miR-221, which might provide a further understanding on the regulation of SOCS1 and SOCS3.
In summary, our findings suggest miR-221 could regulate SOCS1/SOCS3 and functions as an IFN enhancer in HCV infection. Herein, we provide characterization of potential miR-221 target genes and broaden the understanding of IFN stimulated gene regulation.

Materials and methods

Serum specimens, cell lines and reagents

Serum samples used in this study were collected from 34 HCV infected patients with chronic hepatitis (Qujing, Yunnan province, China). The diagnosis was determined by physical examination, blood chemistry, and magnetic resonance imaging. No patient had received chemotherapy. Additional samples were collected from 22 blood donors in Shanghai Changhai Hospital, in which standard markers for HBV or HCV infection tested negative. Clinical and pathological information was extracted from the patients’ medical charts and pathology reports (Table 1). Written consent for tissue donation (for research purposes) was obtained from the patients before serum collection and the protocol was approved by the Institutional Review Board of Second Military Medical University. The samples were frozen in liquid nitrogen and stored at −80 °C until use.

Human embryonic kidney cells (HEK293T) and human HCC cells lines Huh7.5.1 were cultured in DMEM medium plus 10% FBS (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere containing 5% CO₂.

SOCS1 siRNA, SOCS3 siRNA and nonrelative siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Interferon-alpha (IFN-α) was purchased from Piscataway (Middlesex County, NJ, USA); miR-221 mimic, mutant miR-221 mimic and nonrelative mimic control RNA (mm-NC), miR-221 inhibitor and nonrelative inhibitor control RNA (mi-NC) were purchased from Ribobio (Gangzhou, China).

Generation of HCV viral stocks and infection of Huh7.5.1 cells

Infectious HCV in cell culture (HCVcc) was generated as described previously (Lindenbach et al., 2005). Briefly, plasmid pFJ6/JFH1, containing the full-length chimeric genomic cDNA for HCV J6 and JFH-1was linearized and used as the template for transcription using an in vitro MEGAScript kit (Promega). The in vitro-transcribed RNA was delivered to Huh7.5.1 cells by electroporation. Viral stocks were obtained by harvesting the culture supernatants on days 8 to 18 after transfection and aliquoted for storage at −80 °C. The titer of viral stock was determined in Huh7.5.1 cells by immunostaining as described...
previously (Lindenbach et al., 2005). Briefly, HCVC-infected cells were washed, fixed with cold methanol, probed with anti-HCV-positive sera, washed, and probed with FITC-conjugated anti-human IgG (Jackson Immunoresearch, UK). Finally, the cells were labeled with DAPI and imaged in a fluorescence microscope (IX81; Olympus, Tokyo, Japan). Stained foci were counted in quadruplicate wells, and the virus titer of focus-forming units (ffu)/mL was calculated.

Huh7.5.1 cells were seeded in a 24-well plate, and following an overnight culture, a 50 μl HCVC supernatant with 5 × 10^5 ffu/mL was added to each well for 5 h, and then supplemented with fresh culture medium after washing with culture medium 5 times. For miRNA or IFN treatment, 20 nM miR-221 mimic or inhibitor was added onto PVDF membranes (Immobilon P-SQ, Millipore, Billerica, MA, USA). After blocking with 5% nonfat milk, the membranes were incubated with specific primary antibodies at 4°C overnight, followed by incubation with HRP-conjugated goat anti-rabbit or goat anti-mouse antibody (1:10000 dilution, KPL, Gaithersburg, MA, USA) for 1 h at room temperature. Finally, signals were developed with Super Signal West Pico chemiluminescent substrate (Pierce), visualized by the Gene Gnome HR Image Capture System (Syngene, Frederick, MD, USA) and analyzed by Gene tools (Syngene). The primary antibodies used were: HCV core and HCV NS5A (Thermo Fisher, Waltham, MA USA), JAK1, STAT1, STAT3, SOCS1, SOCS3, pJAK1, pSTAT1, pSTAT1 rabbit monoclonal antibody (SAB, College Park, MD, USA) and GAPDH mouse monoclonal antibody (ImmunoGen, Shanghai, China).

**Prediction of miRNAs targets**

TargetScan (http://www.targetscan.org/) and PicTar (http://pic.tar.mdc-berlin.de/) programs were used to investigate the target genes of miRNAs and the conserved sites bound by the seed region of miR-221.

**Statistical analysis**

Data are presented as mean ± SD. Comparisons were made by using a two-tailed t test or one-way ANOVA for experiments with more than two subgroups. P < 0.01 was considered statistically significant.

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**Appendix A. Supporting information**

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.06.024.

**References**


