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**Hepatitis C virus infection, microRNA and liver disease progression**

Shrivastava S *et al.* HCV associated miRNA

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**Abstract**

Hepatitis C virus (HCV) is a global health problem with an estimated 170-200 million peoples (approximately 3% of world population) are chronically infected worldwide and new infections are predicted to be on rise in coming years. HCV infection remains categorized as a major risk factor for chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide. There has been considerable improvement in our understanding of virus life cycle since, the discovery of HCV two-decades ago. MicroRNAs (miRNAs) are important players in establishment of HCV infection and their propagation in infected hepatocytes. They target crucial host cellular factors needed for productive HCV replication and augmented cell growth. Very first anti-miRNA oligonucleotides, miravirsen has been tested in clinical trial and shown promising results as therapeutic agent in treatment against chronic HCV infection. Deregulated expression of miRNAs has been linked to the pathogenesis associated with HCV infection by controlling signaling pathways such as, proliferation, apoptosis and migration. Circulating miRNAs emerging as growing field in identification of biomarkers in disease progression and their potential as a means of communication between cells inside the liver is an exciting area of research in future. This review focuses on recent studies enforcing the contribution of miRNAs in HCV life cycle and coordinated regulation in HCV mediated liver disease progression.

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**Key words:** Hepatitis C virus; MicroRNA; Liver disease; Interferon signaling; Circulatory miRNAs

**Core tip:** Hepatitis C virus (HCV) is the major cause of chronic liver disease that gradually progresses from chronic hepatitis to cirrhosis and hepatocellular carcinoma during the course of infection. MicroRNAs (miRNAs) are small RNA molecules and have the ability to regulate gene expression by targeting mRNA degradation or translational repression. MiRNAs regulate HCV life cycle either by supporting viral replication or by inhibiting interferon signaling pathway. Several miRNAs play important roles in HCV related inflammation, fibrosis and HCC development. This review focuses on the involvement of miRNA in HCV life cycle and virus mediated liver disease progression, emerging role of circulating miRNAs and exploitation of miRNA as alternative therapeutic approach for HCV infection.

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**INTRODUCTION**

Hepatitis C virus (HCV) was first identified as a non-A, non-B hepatitis more than two decades ago[1]. It is a single stranded, positive sense RNA virus belongs to family *flaviviridae* and genus *hepacivirus*. The viral genome encodes for a single precursor polyprotein of approximately 3010 amino acids, which is cleaved by viral and cellular proteases into three structural (core, E1 and E2) and seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) viral proteins. Core protein forms the capsid, which is surrounded by a lipid bilayer containing the glycoproteins, E1 and E2. These viral proteins are responsible for viral replication and various cellular functions [2]. HCV is a major cause of chronic liver disease, mostly asymptomatic in nature. Majority of the infected patients approximately, 80% develop persistent chronic infection and are at high risk for liver cirrhosis and hepatocellular carcinoma (HCC). An estimated 170-200 million peoples worldwide are infected with hepatitis C [3] and about 2.7-3.9 million peoples are living with HCV infection in the United States [4]. In addition, HCC and cirrhosis have been increasing among persons infected with HCV [5]. Recent approval of HCV NS3/4A protease inhibitors in standard treatment, consisting of pegylated interferon (IFN) alpha, and nucleoside analog, ribavirin (RBV) have shown improved rates of sustained virologic response in HCV infected patients [6]. Drugs targeted against HCV polymerase, NS5B has also been successfully validated in phase 2 clinical trials for the treatment of HCV infection [7].

MicroRNAs (miRNAs) were discovered in 1993 during a developmental timing experiment in the nematode *Caenorhabditis elegans*. Till date, human miRNA family has expanded to over 2000 mature miRNAs (miRBase v19.0; <http://www.mirbase.org/>) and *in silico* prediction estimates that approximately 60% of human mRNA could be targets of miRNA [8]. MiRNAs constitute a class of non-coding RNAs, about 18-22 nucleotides long and play crucial role in the regulation of gene expression. The production of miRNAs requires several processing steps, first primary miRNAs (pri-miRNAs) are cleaved by the ribonuclease Drosha to produce precursor miRNAs (pre-miRNAs) which in turn, cleaved by the ribonuclease Dicer to produce mature, single stranded miRNAs [9,10]. Once synthesized, mature miRNA associate with RNA induced silencing complex (RISC) together with Argonaute/EIF2C (AGO) proteins and mediates the target mRNA recognition. MiRNA identify target mRNA through specific base-pairing interactions between the 5' end ("seed" region) of miRNA and sites within coding and untranslated regions (UTRs) especially 3' UTR of mRNAs that lead to mRNA destabilization. MiRNA inhibits the target gene expression either by mRNA degradation or translational repression. MiRNA promotes mRNA cleavage by inducing deadenylation or suppresses protein synthesis by repressing the translation initiation at the cap recognition or inducing ribosomes to drop off prematurely [11,12]. MiRNA biogenesis is beyond the scope of this review and elegant reviews addressing miRNA synthesis and their mechanism of gene regulation is discussed in more detail elsewhere. A combinatorial nature of miRNA regulation, *i.e.*, each miRNA regulates hundreds of different mRNAs and further, a single mRNAs are targeted by multiple miRNAs, will allow us to focus on regulatory networks that determine the cell fate decisions. Viral infection can elicit changes in cellular miRNA expression profile, and several RNA viruses have been reported to interact directly with cellular miRNAs to facilitate their replication potential [13].

**ROLE OF MIRNAS IN HCV REPLICATION**

Recent studies have identified several miRNAs as key players in virus-host interactions, regulating virus replication and pathogenesis during HCV infection. The most abundant miRNA in the liver, miR-122 is regulated by specific, liver-enriched transcription factor, hepatocyte nuclear factor 4α [14] and is responsible for liver homeostasis [15]. Several studies demonstrated that miR-122 is required for HCV replication in infected cells [16-18]. MiR-122 positively modulates HCV infection through direct interactions with viral RNA and stimulates HCV translation [19]. It forms an oligomeric complex in which one miR-122 molecule binds to the 5' UTR of HCV RNA with 3' overhanging nucleotides, masking the 5' terminal sequences of HCV genome. Furthermore, specific internal nucleotides as well as 3' terminal nucleotides in miR-122 were absolutely required for maintaining HCV RNA abundance [20]. MiR-122 recruits Argonaute 2 to the 5' end of the viral genome, stabilizing the viral RNA and avoid the degradation in infected cells [21]. Recent study also demonstrated that miR-122 protects HCV RNA from 5' decay by targeting 5' exonuclease Xrn1 [22]. Exogenous expression of miR-122 allows efficient HCV RNA replication and/or infectious virion production in non-permissive cell line [23-25]. Apart from regulating viral replication, miR-122 is also involved in cell cycle progression in hepatoma cell line [26]. MiR-122 is known to target cyclin G1 and use of miR-122 inhibitor has been reported to prevent the alcohol-induced increase in HCV RNA and protein levels [27].

Besides miR-122, other miRNAs have been involved in HCV replication. Overexpression of miR-448 and miR-196 were able to substantially attenuate viral replication by directly targeting CORE and NS5A coding region of the HCV genome, respectively [28]. let-7b was also identified as novel cellular miRNAs that directly target HCV genome and elicits anti-HCV activity [29]. Mutational analysis identified let-7b binding sites at the coding sequences of NS5B and 5'-UTR of HCV genome that were conserved among various HCV genotypes. Overexpression of miR-199a inhibited HCV replication in cells bearing HCV-1b or -2a genome length replicon [30]. MiR-196a inhibits HCV RNA and NS5A protein expression in replicon by regulating HMOX1/Bach1 expression [31]. In HCV infected patients, lower expression levels of miR-29 was observed in liver and overexpression of miR-29 inhibits viral RNA in HCV infected hepatocytes [32]. We have demonstrated that miR-130a expression is upregulated in liver biopsy from HCV infected patients as well as in HCV infected hepatocytes *in vitro* [33]. We also observed that knockdown of miR-130a inhibits HCV replication in hepatocytes. Similar observation has been reported on miR-130a mediated regulation of viral replication in HCV infected cells [34]. Differential upregulation of hsa-miR-130a, hsa-miR-130b, hsa-miR-298, hsa-miR-193a-5p and hsa-miR-371-5p were also observed in HCV Con1 replicon in comparison to control cells. These miRNAs have been associated with cell growth by targeting genes PPARG, IRF1 and STAT3 in HCV infected cells [35]. Differential expression of miRNAs such as, miR-24, miR-149, miR-638 and miR-1181 were also identified following HCV infection and are involved in HCV entry, replication and propagation [36]. Delivery of miR-17-92 cluster has been reported to inhibit HCV replication by up to 95% *in vitro* cell culture system [37]. Recently, negative effect of miR-27a has been demonstrated in HCV replication. miR-27a repression increased the cellular lipid content, decreased the buoyant density of HCV particles and increased viral replication and infectivity [38]. MiR-192/miR-215 and miR-491 are capable of enhancing HCV replication in replicon cells [39]. MiR-141 mediated suppression of DLC-1 (a Rho GTPase-activating protein) enhances viral replication in HCV-infected primary human hepatocytes [40].

**ROLE OF MIRNAS IN REGULATION OF INTERFERON RESPONSE IN HCV INFECTION**

HCV infection also modulates several miRNAs, which in turn inhibits type 1 IFN signaling pathway. We have demonstrated that HCV inhibits IFITM1, an interferon stimulated gene, by upregulating miR-130a expression in HCV-infected hepatocytes. Introduction of anti-miR-130a in hepatocytes increased IFITM1 expression with concomitantly reduction in HCV replication [33]. Overexpression of miR-122 has also been associated with inhibition of IFN signaling pathway. Silencing of miR-122 enhances IFN-induced ISRE activity, by decreasing expression of SOCS3. This decrease in SOCS3 level was also regulated by enhanced methylation at SOCS3 gene promoter, implicating additional mechanism of inhibition of HCV replication using antisense oligonucleotides of miR-122 [41]. miRNAs also regulate the expression of target genes involving immune response to viral infections mediated by type I IFN pathway. Upregulated miR-21 suppressed MyD88 and IRAK1 expression in hepatocytes, which subsequently repressed type I IFN effector gene expression and the type I IFN-mediated antiviral response, thereby promoting viral replication [42]. IFN-α treatment also modulates HCV-specific miRNAs expression in hepatocytes. miR-324-5p and miR-489 shown to be upregulated in the presence of IFN-α while differential expression of miR-30c and miR-130a were observed between HCV-infected Huh7.5 cells treated with or without IFN-α [34]. MiR-30 cluster targets SOCS1 and SOCS3 genes that act as negative regulators of cytokine signaling. Specifically, SOCS1 and SOCS3 inhibit JAK tyrosine kinase activity and STATs in the JAK-STAT signaling pathway suggesting that IFN-α induced miRNAs modulates gene expression in HCV infected hepatocytes [34]. IFN-β treatment of Huh-7 cells showed an upregulation of miR-142-3p and miR-128a, and these miRNAs were downregulated in HCV replicon-expressing cells [43]. IFN-β induced miRNAs, in conjunction with the downregulation of miR-122, was also studied to prevent HCV replication. Introduction of anti-miRs against miR-196, miR-296, miR-351, miR-431 and miR-448, with and without the inclusion of miR-122 mimic, attenuated the IFN-β mediated reduction of viral RNA by ~75% [28]. Treatment with a toll-like receptor-7 (TLR-7) agonist, imiquimod, downregulates miR-146a and miR-155 in PBMCs from HCV infected patients as compared to their expression in PBMCs of healthy individuals [44].

Increasing evidence also suggests that miRNAs have a profound impact on host defense to HCV infection and clinical outcome of standard HCV therapy. miRNA expression profiles were examined to identify the miRNAs associated with the standard treatment (IFN-α with ribavirin) to CHC patients. Expression levels of 9 miRNAs were significantly different in the sustained virological response (SVR) and non-responder (NR) groups, suggesting that expression pattern of these hepatic miRNA are associated with therapeutic outcome in CHC patients [45]. The expression level of miR-122 was reportedly associated with early response to IFN treatment. HCV infected patients who did not respond to therapy had significantly lower miR-122 levels as compared to responder [46].

**ROLE OF MIRNAS IN HCV RELATED INFLAMMATION AND FIBROSIS**

Many miRNAs have been implicated in various cancers either as oncogenes or tumor suppressor genes. HCV infection induces chronic inflammation and regulation of inflammation related miRNA favors the initiation and progression of HCC. Gene expression analyses identified dysregulation of miR-449a in HCV patients but not in alcoholic and non-alcoholic liver diseases. YKL40 is an inflammatory marker known to be upregulated in patients with chronic liver diseases with fibrosis and miR-449a regulates the expression of YKL40 by targeting NOTCH signaling pathway following HCV infection [47].

In patients infected with HCV, miR-155 expression levels were markedly increased, and promote hepatocyte proliferation and tumorigenesis by modulating Wnt signaling [48]. Chronic HCV infection induced liver fibrosis is mediated by upregulation of transforming growth factor (TGF)-β [49]. In HCV-infected patient samples and in a mouse carbon tetrachloride fibrosis model, expression levels of miR-21 were positively correlated with fibrotic stage [50]. MiR-21 was shown to target SMAD7, a negative regulator of TGF-β signaling, leading to increased fibrogenesis [50]. Inhibition of miR-29 was also linked with activation of hepatic stellate cells and collagen synthesis [32].

**ROLE OF MIRNAS IN HCV RELATED HCC**

HCC is often considered as a complication of chronic liver disease, comprises of a single group, regardless of the etiology of liver disease. There are several risk factors associated with development of HCC, including chronic hepatitis C infection [51]. Progression towards HCC involves multiple steps that ultimately lead to deregulation of various signaling pathways and help host cells to acquire metastatic potential in presence of surrounding microenvironment [52]. Chronic hepatitis C is a major risk factor associated with HCC [53]. HCV encoded viral proteins both singly or in coordinated manner, interact with host cellular factors and regulate signaling pathways such as, cell proliferation and apoptosis for augmentation of hepatocyte growth that may contribute towards HCC progression [54]. miRNA dysregulation has been linked with initiation and progression of HCC [55-57], however, the role of miRNAs in HCV-related HCC is poorly understood. The identification of HCC related miRNA signatures is of great value for the early diagnosis of HCC, before the onset of disease in HCV-positive patients. Limited studies are available addressing the role of miRNA expression in HCV associated HCC. Differential miRNA expression from formalin fixed paraffin embedded HCV infected HCC specimens indicated 10 upregulated and 19 downregulated miRNAs [58]. Another study in HCV infected patients, 13 miRNAs were shown to be downregulated and were predicted to target genes related to immune response, antigen presentation, cell cycle, proteasome, and lipid metabolism signaling pathways [59]. However, validation of these miRNAs and their predicted targets are necessary for conclusive role of particular miRNA in HCV related HCC. A list of altered miRNAs associated with HCV infection and their proposed role in liver disease progression has been summarized in Table 1.

**CIRCULATORY MIRNAS IN HCV INFECTION**

One of the major challenges in HCV research is early detection of liver disease which allow us for rapid intervention and improved outcome of antiviral treatment. Liver biopsy is often recommended in patients with unexplained elevated serum aminotransferases in order to determine the cause, grade of hepatic inflammation and stage of hepatic fibrosis. Non-invasive or minimally invasive methods need to be developed which can evaluate disease severity and the likelihood of disease progression. Circulating miRNAs have been demonstrated to be very specific and stable in human serum and plasma. In addition, circulating miRNAs display consistent profiles between healthy individuals and significantly altered levels in disease conditions [60, 61]. These characteristics of circulating miRNAs established their potential value as biomarkers for detection and as predictive marker for liver disease progression in HCV infection. We have performed serum/plasma specific miRNA array and observed that several circulating miRNAs are significantly upregulated in sera of HCV infected patients as compared to healthy controls [62]. We have shown that increased expression of miR-20a and miR-92a is specific to HCV associated liver disease because we did not observe an upregulation of these miRNA in sera of patients with non-HCV related liver disease. Subsequently, we observed that elevated levels of miR-20a were positively correlated with disease severity in HCV infected patients, however, miR-92a expression is reduced with higher grade of fibrosis in HCV infected patients [62]. miRNA profiling was also performed to identify the expression of 940 human miRNAs in the serum of HCV infected individuals. Serum levels of miR-134, miR-320c and miR-483-5p were significantly upregulated in HCV infected patients [63]. Serum levels of miR-122 were correlated with disease parameters in patients with CHC by several groups. The higher levels of miR-122 and miR-192 was observed in sera from patients with CHC and in other etiologies associated with liver injury as compared to sera from healthy controls [64-68]. The serum level of miR-122 and miR-21 strongly correlates with serum alanine leucine transaminase levels and higher necroinflammatory activity in the liver in patients with CHC infection suggesting their potential as a serum biomarker over ALT in predicting the presence of chronic HCV infection [64, 65, 68, 69], although the specificity of miR-122 for HCV mediated liver disease is questionable. Levels of miR-125b and miR-146a were also increased in the serum of CHC patients compared to healthy controls [70]. Treatment-naïve patients with chronic HCV infection have been shown to have higher expression of miR-155 in their circulating monocytes as compared to individuals who cleared HCV infection after therapy, suggesting a possible correlation between increased miR-155 and HCV viral presence and/or replication [70].

Circulating miRNAs in urine were also examined for developing screening methods. Expression of 3 upregulated miRNAs, miR-625, miR-532 and miR-618, were evaluated as non-invasive biomarkers for the early diagnosis of HCC among high-risk HCV positive patients. Elevated expression of miR-625, miR-532 and miR-618 were observed in 56%, 62.5% and 72% of HCC-post HCV positive patients, respectively. In addition, miR-516-5p and miR-650 were found to be down-regulated in 50% and 72% of HCC-post HCV positive patients, respectively. Differential expressions of these miRNAs were predicted to possibly target genes related to HCC development and progression in high risk HCV patients [71]. The function of these extracellular circulating miRNAs is not well understood. The liver is a complex organ where various cell types reside and interact in close vicinity. During HCV infection, there could be multiple factors that contribute to release of miRNAs in the circulation. Secretion of miRNA from different cell types in a cell-specific manner in response to HCV infection cannot be ruled out. Further studies are warranted to investigate the cellular source of circulating miRNAs.

**MIRNA AS THERAPEUTICS IN HCV INFECTION**

Therapies that target essential host factors required for HCV replication could present an effective approach for the development of new HCV antiviral drugs. Therapeutic potential of miR-122 employing antisense oligonucleotide (SPC3649) complementary to the 5’-end of miR-122, has been evaluated in HCV infected chimpanzees [72]. SPC3649 therapy resulted in a reduction of HCV viral load in the liver and blood of chronically infected chimpanzees. In addition, reduction in viral load was accompanied by normalization of the endogenous interferon pathway, which is maximally induced in chronically infected chimpanzees, suggesting the restoration of host immune response following treatment with SPC3649 [72]. Recently, miravirsen (a locked nucleic acid-modified antisense oligonucleotide for miR-122) showed prolonged dose-dependent reductions in HCV RNA levels without evidence of viral resistance in chronic HCV genotype 1 infected patients in a Phase IIa clinical trial [73]. No adverse side effects of miR-122 inhibition have been documented in either chimpanzees or chronic HCV infected patients. Additional host cell factors that help HCV for productive replication such as, cyclophilin A and phosphatidylinositol-4-kinase III alpha have emerged as a promising alternative [74]. The efficacy of anti-miR-122 along with current anti-HCV drugs needs to be evaluated in future trials that could provide better therapy outcome in terms of lesser rate of relapse, interferon free regimens and reduced possibility of drug resistance. miRNAs function either as oncomirs or tumor suppressors are involved in cell growth regulatory pathways, have rapidly emerged as targets for therapeutics in the pathogenesis of HCV infection. Successful delivery of either miRNA mimics to restore the activity of tumor suppressor miRNAs or anti-miR oligonucleotides for pharmacological inhibition of oncogenic miRNAs, understanding of potential off-target effects and physiologic consequences of long-term miRNA modulation *in vivo* are some of the important factors to keep into consideration in future development of miRNA therapeutics.

**CONCLUSION**

We are still in the infancy stage of understanding the potential of manipulating miRNAs for the treatment of HCV infection. HCV infection modulates a set of miRNAs that regulate host immune response and cell growth interconnected with multiple signaling pathways. MiRNA mediated regulation of gene expression will help us to understand the signaling pathway and disease progression associated with HCV infection. Therapeutic silencing of miR-122 opens a door for novel drugs and therefore, identification of microRNAs with a prominent role in HCV viral life cycle and its implication in liver disease progression is emerging as a therapeutic option against chronic HCV infections. A major drawback of exploiting miRNA as therapy may have adverse side effects because of the biological properties of miRNA where, a single miRNA binds to multiple targets and regulate various signaling pathways simultaneously. On the contrary, recent studies on circulating miRNAs generates an alternative approach for identification of minimally invasive biomarker for HCV mediated liver disease and as predictive biomarker to categorize patients those may develop end stage liver disease due to viral infection. Indeed, further in-depth studies are needed to identify mechanistic insights behind modulated miRNAs by HCV infection.

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