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MINIREVIEW

The Role of MicroRNAs in Hepatitis C Virus Replication and Related Liver Diseases

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Hepatitis C virus (HCV) infection is a worldwide health problem and is one of the main causes of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). However, only limited therapeutic options and no vaccines are currently available against HCV infection. Recent studies of microRNAs (miRNAs), which are able to regulate HCV replication and its related liver diseases by directly interacting with the HCV genome or indirectly controlling virus-associated host pathways, have broadened our understanding of the HCV life cycle. HCV utilizes host cellular miRNAs and modulates expression of miRNAs in infected hepatocytes for its infection and propagation. Moreover, such miRNAs directly or indirectly alter HCV replication efficiency and induce liver diseases including liver fibrosis, cirrhosis, or HCC. Representatively, miR-122 directly modulates the HCV life cycle by increasing HCV translation and genomic RNA stability. Recently, a phase IIa clinical trial with miravirsen, an LNA form of antimiR-122 oligonucleotides, showed significant reduction in serum HCV levels in patients chronically infected with HCV with no detectible evidence of resistance. In addition to miR-122, other miRNAs involved in the regulation of HCV propagation could be targeted in strategies to modulate HCV replication and pathogenesis. In this review, we summarize the features of miRNAs critical for HCV replication and HCV-mediated liver abnormalities and briefly discuss their potential application as therapeutic reagents for the treatment of HCV infection and its related diseases.

***Keywords*:** hepatitis C virus, liver disease, microRNA, therapeutics, antagonist, mimics

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Introduction

Hepatitis C virus (HCV) is a hepatotropic, single-stranded, positive sense RNA virus belonging to the family *Flaviviridae* and genus *Hepacivirus* (Takamizawa *et al.*, 1991). The approximately 9.6-kb length of the HCV genome contains a single open reading frame (ORF) for a precursor polyprotein that is co- or post-translationally processed to the three structural (core, E1, and E2) and seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins responsible for viral replication and various cellular functions (Moradpour *et al.*, 2007). The polyprotein ORF is flanked by the 5′ and 3′ untranslated regions (UTR), both of which are important *cis*-elements for the replication and translation of HCV (Liu *et al.*, 2009; Lohmann, 2013). The 5′ UTR contains the internal ribosome entry site (IRES) that recruits ribosomes to initiate the translation of the HCV genome into a single polyprotein (Kim and Chang, 2013). In addition, sequence elements and secondary structures in the 5′ UTR and core coding region are involved in the replication of HCV (Friebe *et al.*, 2001; Vassilaki *et al.*, 2008). The 3′ UTR consists of a specific tripartite structure - a variable region, a poly(U/UC) tract of variable length, and a highly conserved X tail - required for efficient HCV RNA replication (Kolykhalov *et al.*, 1996; Blight and Rice, 1997; Georgel *et al.*, 2010). Although the detailed mechanism by which the 3′ UTR acts on RNA replication is not clear, elements in the 3′ UTR in concert with two other *cis*-acting replicative elements (CREs) within the NS5B coding region were revealed to be essential for HCV replication in cell culture as well as *in vivo* (Kolykhalov *et al.*, 2000; Yi and Lemon, 2003; Lohmann, 2013).

In addition to the viral *cis*- and *trans*-factors, HCV utilizes host components to enhance the translation and replication of its genome. A variety of cellular (micro)RNAs and RNA binding proteins are recruited by the sequence and structural elements of HCV 5′ and 3′ UTRs for its efficient translation and replication (Hoffman and Liu, 2011; Niepmann, 2013). One of the most interesting discoveries is that the liver-specific microRNA-122 (miR-122) positively regulates HCV infection through direct interactions with the viral RNA genome and facilitates HCV RNA accumulation and translation (Jopling *et al.*, 2005; Niepmann, 2009; Shimakami *et al.*, 2012b). Typically, microRNAs (miRNAs) regulate eukaryotic gene activity at the post-transcriptional level through specific base-paring interactions with the complementary sites within coding or 3′ UTR regions of mRNA (Huntzinger and Izaurralde, 2011; Fabian and Sonenberg,

2012). The production of miRNAs requires a series of processing steps that converts the primary miRNA transcripts (pri-miRNA) to the biologically active, mature miRNA (Kim *et al.*, 2009; Winter *et al.*, 2009). First, pri-miRNAs are recognized and cleaved by a microprocessor complex composed of the RNase III-like enzyme Drosha and dsRNA-binding protein DGCR8 to yield precursor miRNAs (pre-miRNAs) in the nucleus (Lee *et al.*, 2003; Han *et al.*, 2004). Then, pre-miRNAs are transferred to the cytoplasm via the Exportin 5 pathway and cleaved by another RNase III-like enzyme Dicer to produce ~22-bp mature miRNA duplexes with 3′-overhangs (Lee *et al.*, 2002; Kim, 2004). One of the strands of the duplex is associated with RNA-induced silencing complex (RISC) and is used as a sequence-specific guide to recognize and bind to its target mRNA(s) (Huntzinger and Izaurralde, 2011; Fabian and Sonenberg, 2012). This process induces inhibition of the expression of target mRNA(s) either by mRNA degradation or translational repression depending on the extent of base pairing between the miRNA and the mRNA target. When the miRNA perfectly matches its target, Ago2, as part of the RISC, cleaves the target mRNA, resulting in degradation of the target mRNA. On the contrary, when imperfect base pairing between the miRNA and target mRNA occurs, the interaction of the RISC with the target mRNA generally mediates repression of translation (Filipowicz *et al.*, 2008; Guo *et al.*, 2010).

The human genome expresses over 2,500 miRNAs (www.mirbase.org, released June 2013) and it is estimated that each can regulate as many as 1,500 genes and each mRNA is likely to be regulated by several miRNAs (Hafner *et al.*, 2010). Thus, the interaction of miRNAs and their target mRNAs makes up a complex gene regulatory network that is involved in virtually every cellular process, including cell growth, development, metabolism, differentiation, disease pathology, and antiviral defense (Esquela-Kerscher and Slack, 2006; Stefani and Slack, 2008; Witwer *et al.*, 2010). With regard to virus infection, increasing evidence suggests that miRNAs or other machineries of the miRNA pathway can influence viral replication and its related diseases at multiple levels (Sarnow *et al.*, 2006; Gottwein and Cullen, 2008). Moreover, viral infection can bring out changes in the cellular miRNA expression profile and even viruses themselves can encode miRNAs to modulate their replication and virus-associated host pathways. Furthermore, some RNA viruses, such as HCV, directly interact with cellular miRNAs to regulate their replication (Roberts *et al.*, 2011b).

In this review, we describe several miRNAs that regulate HCV replication either directly through interaction with viral genomes or indirectly through regulation of virus-associated host pathways. In addition, we summarize miRNAs that are regulated by HCV, emphasizing their role in viral replication and its related liver disease progression. Finally, we briefly discuss miRNA antagonists or miRNA mimics as potential therapeutic options against HCV infection and HCV-mediated diseases.

miRNAs that modulate HCV replication through direct interaction with HCV RNA

The single-stranded, positive sense HCV genome has two functions: it serves as a template for viral replication and as mRNA for the expression of viral proteins. Therefore, host RNAs such as miRNAs and several cellular RNA binding proteins can modulate the replication and translation of HCV through direct binding with HCV RNA (Hoffman and Liu, 2011; Niepmann, 2013). The first evidence of the relationship between miRNAs and HCV infection was reported by Jopling *et al.* (2005). They demonstrated that the liver-specific miRNA, miR-122, stimulates propagation of HCV RNA rather than repressing it, which was a breakthrough finding that coupled a host miRNA to a human infectious disease for the first time (Conrad and Niepmann, 2014). Further studies revealed that miR-122 binds directly to the 5′ UTR of the HCV genome at two adjacent sites (Jopling *et al.*, 2008; Machlin *et al.*, 2011), stimulates HCV translation (Roberts *et al.*, 2011a; Henke *et al.*, 2014), and promotes HCV RNA accumulation by stabilizing the HCV genome (Shimakami *et al.*, 2012b). Binding of miR-122 to the 5′ UTR of HCV RNA with 3′ overhanging nucleotides was hypothesized to mask and protect the 5′ end of the viral genome from 5′ decay mediated by 5′ exonuclease Xrn1 (Li *et al.*, 2013b). Even the exogenous expression of miR-122 fa-

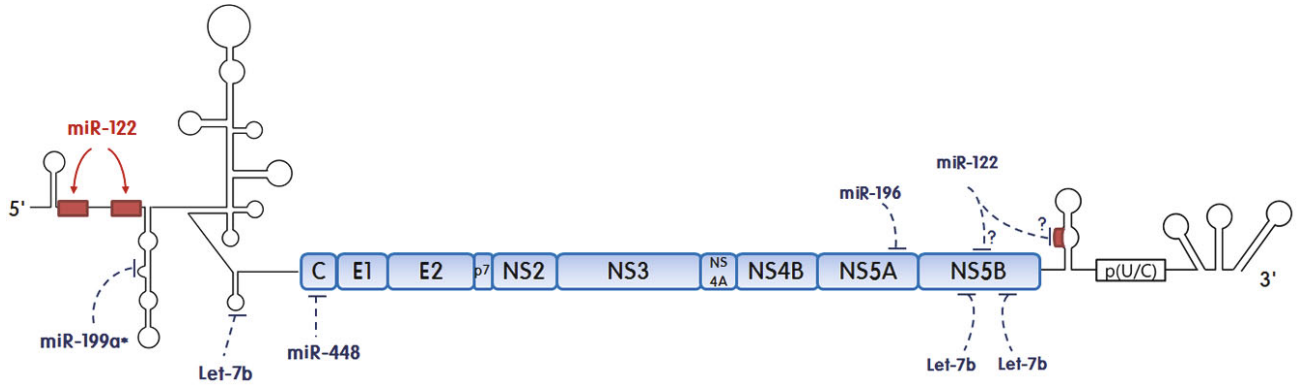

Fig. 1. miRNAs that modulate HCV replication through direct interaction with HCV RNA genome. miRNAs positively regulating HCV replication are indicated in *red*. Inhibitory miRNAs are indicated in *blue*.

cilitates HCV replication and supports the entire HCV life cycle in non-permissive hepatic and non-hepatic cell lines (Narbus *et al.*, 2011; Fukuhara *et al.*, 2012; Kambara *et al.*, 2012).

In addition to miR-122 itself, Dicer and TRBP, which process pre-miR-122 into mature miR-122, are required for the activation of HCV replication (Zhang *et al.*, 2012a). Ago2 is also required for the miR-122 regulation of HCV RNA accumulation and translation (Wilson *et al.*, 2011; Shimakami *et al.*, 2012a). Localization of Ago2 to the HCV replication complex suggests its possible role in the subsequent steps of miR-122 activity (Ariumi *et al.*, 2011). The HCV genome harbors two more miR-122 target sites, one in the variable region of the 3′-UTR and the other in the NS5B coding region (Fig. 1). However, the role of the potential miR-122 target site in the 3'-UTR is currently controversial (Nasheri *et al.*, 2011; Shimakami *et al.*, 2012b; Conrad *et al.*, 2013); thus, more work is required to resolve its full contribution. In the case of the target site in the NS5B coding region, miR-122 was reported to inhibit HCV replication through binding to this site in the replicon system, suggesting that this binding site probably functions as a repressor for HCV replication (Nasheri *et al.*, 2011).

Besides miR-122, other miRNAs have also been reported to interact directly with HCV RNA (Fig. 1). Overexpression of miR-199a* inhibits HCV genome replication in HCV 1b or 2a cell lines through binding to the stem-loop (SL) II region of HCV 5′ UTR (Murakami *et al.*, 2009). Since miR-199* appears to be expressed at low levels in liver tissue compared to other tissues (Liang *et al.*, 2007), it would be an another determinant for the liver tropism of HCV (Pietschmann, 2009). In addition to miR-199*, let-7b was identified, which directly targets the NS5B coding region and 5′ UTR sequences of the HCV genome, resulting in a decrease in HCV replicon replication, down-regulation of HCV accumulation, and reduction of HCV infectivity (Cheng *et al.*, 2012). Two other microRNAs, miR-448 and miR-196, directly target the core and NS5A coding region of the HCV genome, respectively (Fig. 1). Overexpression of these two miRNAs substantially reduces HCV replication (Pedersen *et al.*, 2007). Interestingly, interferon (IFN) enhances expression of miR-196 (Hou *et al.*, 2010; Bruni *et al.*, 2011), while HCV infection represses miR-196 expression. More work is needed to determine whether there is an additional direct or indirect involvement of miR-196 in HCV replication.

miRNAs that modulate HCV replication through regulating host signaling pathways

The type I IFN response protects cells against invading viral pathogens (Takaoka and Yanai, 2006). To overcome its antiviral action, HCV infection modulates several miRNAs that are able to regulate the type I IFN signaling pathways (Fig. 2). HCV infection induces expression of miR-130a, which in turn inhibits an IFN stimulated gene, IFITM1, with concomitant efficient HCV RNA replication (Bhanja Chowdhury *et al.*, 2012). However, other studies found that miR-130a inhibits HCV replication through restoration of the host innate immunity in TLR3- and RIG-I deficient cells (Li *et al.*, 2013a). Therefore, the actual role of miR-130a in HCV infection remains to be determined.

In addition to direct binding to the HCV genome, miR-122 has also been associated with suppression of the IFN signaling pathway. Interestingly, Yoshikawa *et al.* (2012) reported that overexpression of miR-122 represses the activity of the IFN-stimulated response element (ISRE). On the contrary, silencing of miR-122 enhances ISRE activity by decreasing expression of SOCS3, leading to an increase in anti-HCV activity (Yoshikawa *et al.*, 2012). This study indicates an additional mechanism of the regulation of HCV replication by miR-122.

Besides miR-122, miR-196b, which inhibits HCV replication by directly binding to the 5' UTR of the HCV genome, also modulates HCV replication by regulating the activity of IFN (Scagnolari *et al.*, 2010). miR-196b suppresses the expression of Bach1, a transcriptional repressor of heme oxygenase 1 (HMOX1) (Hou *et al.*, 2010). HMOX1 has a key anti-inflammatory and anti-oxidant activity and its resulting derepression inhibits HCV replication. HCV infection

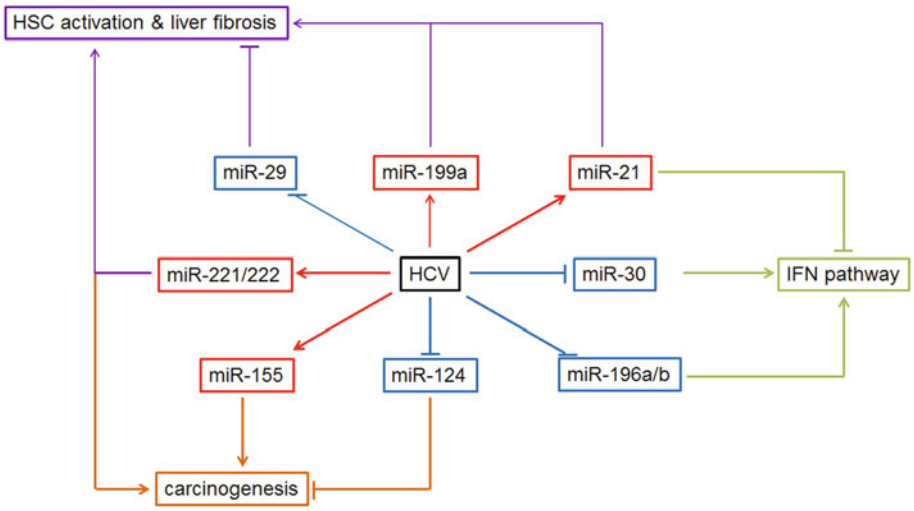

Fig. 2. Altered expression of miRNAs in association with HCV infection and their involvement in HCV-induced liver disease progression. miRNAs that regulate the IFN signaling pathway and liver fibrogenesis are depicted in *green* and *purple*, respectively. miRNAs associated with HCC progression are indicated in *orange*.

also induces expression of miR-21, which targets and inhibits NF-kB (Marquez *et al.*, 2010b), MyD88, and IRAK1 (Chen *et al.*, 2013), essential components of IFN signaling, thereby promoting viral replication.

IFN-α/β treatment also modulates expression of HCV-specific miRNAs such as the miR-30 cluster, miR-342-5p, miR-489, miR-142-3p, and miR-128a in hepatocytes (Bruni *et al.*, 2011; Zhang *et al.*, 2013). Among these, the miR-30 cluster targets SOCS1 and SOCS3 genes that serve as negative regulators of cytokine signaling.

In addition to innate immune response, some miRNAs modulate other signaling pathways for HCV propagation. During infection, HCV can alter the microRNA expression pattern of the cell to facilitate its replication. Liu *et al.* (2010) identified 42 microRNAs that had an altered expression pattern upon HCV infection, of which 22 were up- and 20 down-regulated. However, the role of these differentially expressed miRNAs has been identified in only a few of them so far.

HCV infection up-regulates miR-320c and miR-483-5p. These miRNAs have putative targets in PI3 kinase/AKT signaling, MAP kinase, and the NF-kB pathway, which are known to regulate cell proliferation, apoptosis, or the inflammation response (Shwetha *et al.*, 2013). Similarly, miR-491, a microRNA that is also up-regulated by HCV infection, has been known to enhance HCV replication possibly through suppression of the PI3 kinase/AKT signaling pathway (Ishida *et al.*, 2011; Hoffmann *et al.*, 2012).

Contradictory results have been reported for miR-27a. One study reported that miR-27a reduces the cellular triacylglyceride and cholesterol content (Shirasaki *et al.*, 2013), and the other reported that miR-27a increases the cellular lipid content (Singaravelu *et al.*, 2013). However, in either case, miR-27a inhibits HCV replication and infectivity. Nevertheless, HCV induces the expression of miR-27a (Shirasaki *et al.*, 2013; Singaravelu *et al.*, 2013). It is speculated that up-regulation of miR-27a by HCV may contribute to escape from host immune surveillance and establishment of a persistent chronic HCV infection (Singaravelu *et al.*, 2013). In contrast, miR-27b enhances production of extracellular infectious HCV. This finding may be related to the increase in cellular lipid content (Shirasaki *et al.*, 2013). However, whether expression of miR-27b can be modulated by HCV is controversial (Steuerwald *et al.*, 2010). Taken together, the regulation mechanism of miRNAs by HCV and their roles in the various steps of the HCV life cycle with regard to the host cellular signaling pathways await further investigation.

miRNAs that modulate HCV-induced liver disease and HCC

Chronic hepatitis C is a major risk factor associated with hepatocellular carcinoma (HCC) (Raimondi *et al.*, 2009). Recent studies have demonstrated that HCV-induced modulation of miRNAs favors the initiation and progression of HCV-related liver diseases such as fibrosis, cirrhosis, and HCC (Fig. 2). Up-regulation of miR-155 in HCV-infected patients promotes proliferation and progression to HCC by modulating Wnt signaling (Zhang *et al.*, 2012b).

HCV infection induces chronic inflammation and increases transforming growth factor (TGF)-β signaling, promoting liver fibrosis (Zampino *et al.*, 2013). In HCV-infected patients, expression levels of miR-21 are positively correlated with the fibrotic stage (Marquez *et al.*, 2010a). HCV infection has been shown to increase the expression of miR-21 and directly represses Smad7, a negative regulator of TGF-β signaling, leading to the promotion of fibrogenesis (Marquez *et al.*, 2010a).

miR-124 is a putative tumor suppressor and its expression is frequently reduced in HCC. HCV core protein reduces miR-124 expression, which promotes migration and invasion of HCV-related intrahepatic cholangiocarcinoma cells (HCV-ICC) (Zheng *et al.*, 2012). miR-124 exerts its effects through a mechanism that involves modulation of SMYD3, a regulator of the oncogenes c-Myc and MMP9 (Zeng *et al.*, 2012).

Expression of miR-199-5p/miR-199-3p or miR-221/222 is correlated with fibrosis in HCV infected patients (Murakami *et al.*, 2011). High expression of miR-221 and 222 was reported to induce fibrosis through activation of hepatic stellate cells (Ogawa *et al.*, 2012) and carcinogenesis via down-regulation of the tumor suppressor genes p27, p57, and PTEN (Fornari *et al.*, 2008; Pineau *et al.*, 2010). Another study found 10 up-regulated and 19 down-regulated miRNAs in HCV-infected HCC specimens (Varnholt *et al.*, 2008). However, their predicted targets need to be validated to dissect the roles of particular miRNAs in HCV-related HCC.

miRNAs as therapeutics against HCV infection

The main treatment option for HCV infection is a combination of pegylated IFN-α (PEG-IFNα) and ribavirin. This treatment clears genotype 2 and 3 virus infection in up to ~85% of cases, but in the case of genotype 1, only ~45% are able to support a sustained viral response (Fried *et al.*, 2002). Recent approval of two direct-acting antivirals (DAA) targeting HCV NS3 protease, telaprevir (VX-950) and boceprevir, gives hope for the clearance of HCV infection, but still these drugs are combined with PEG–IFNα and ribavirin and are prone to the appearance of drug resistant viruses (Kieffer *et al.*, 2010).

Regarding the essential and multifunctional roles of miRNAs for HCV infection, miRNA mimics or miRNA antagonists could represent an effective approach for the development of new anti-HCV drugs (Lee *et al.*, 2013). Recently, a phase 2 clinical trial with SPC3649 (formerly Miravirsen), LNA-modified antisense oligonucleotides directed against miR-122, was completed for the treatment of HCV and showed a dose-dependent prolonged reduction of up to 2-3 logs of viral RNA in patients chronically infected with HCV genotype 1 (Janssen *et al.*, 2013). Treatment of HCV-infected cells with miR-196b and miR-29 is known to reduce HCV replication efficiency (Pedersen *et al.*, 2007; Bandyopadhyay *et al.*, 2011). IFN-β-induced miRNAs, in combination with the downregulation of miR-122, was also studied to prevent HCV replication (Pedersen *et al.*, 2007). With more information on miRNA regulatory networks and their roles in

HCV-induced disease processes, we can surely develop and possess numerous miRNA targeting therapies in the near future.

Conclusion and future perspectives

Alterations in the miRNA expression profile induced by HCV infection have been reported to modulate HCV propagation and HCV-related liver diseases. These miRNAs regulate HCV infection through direct binding to the HCV genome or indirect alteration of the host immune response and cell growth interconnected with multiple signaling pathways. Although high-throughput screening methods have revealed a complicated network of regulation, further studies to dissect the complex networks between miRNAs and HCV infection will help us to understand the signaling pathways and disease progression associated with HCV infection. In this aspect, therapeutic silencing of miR-122 is opening a new era for HCV therapy. Targeting specific miRNAs with miRNA mimics or antagonists is reasonably simple as it only requires change and/or modification of nucleotide sequences, and thus it is theoretically practical against all kinds of diseases involving abnormal expression of miRNAs. Therefore, miRNAs with a critical role in the HCV life cycle or HCV-induced liver disease progression are emerging therapeutic targets or agents against chronic HCV infection.

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