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MicroRNA-122: a Therapeutic Target For Hepatitis C Virus (HCV) Infection

Giuseppe Nunnari[1,2], Matthias J Schnell[2]

[1]Department of Medicine and Medical Specialties, Division of Infectious Diseases, University of Catania, Catania, Italy, [2]Department of Microbiology and Immunology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, USA

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1. ABSTRACT

Hepatitis C virus infection is the main cause of liver disease worldwide, often leading to chronic hepatitis. Recent studies have demonstrated that miRNA-122, a liver-specific miRNA, is required for HCV replication in hepatocytes by its binding to the 5' UTR of HCV. Down-regulation of miRNA-122 *in vitro* and *in vivo* has led to significant inhibition of viral replication. In the present article, we report the major recent findings on the potential therapeutic role of anti-miRNA-122 molecules.

2. INTRODUCTION

Hepatitis C virus (HCV) is a 9.6 kb positive-strand RNA virus of the *Flaviviridae* family. It is the leading cause of liver infection worldwide with over 170 million infected individuals. HCV infection often leads to chronic liver disease, with different degrees of liver damage ranging from fibrosis to liver cirrhosis, with an increased risk of liver failure and hepatocellular carcinoma (HCC) (1).

The current standard therapy for HCV infection is based on interferon alpha (IFN-alpha) and ribavirin,

which has a success rate of 50% in HCV-infected individuals carrying genotype 1 (1). The efficacy of new anti-HCV drugs targeting HCV protease and polymerase is currently under investigation, although the emergence of viral resistance to such drugs is leading the field to study new therapeutic approaches that tackle viral or host factors (2,3). The latter are considered excellent targets because they are likely to have a higher barrier to resistance compared to the highly flexible viral genome.

Several studies on micro RNA (miRNA) and HCV infection and replication, both *in vitro* and *in vivo*, have reported that miRNA-122 plays a key role in HCV replication (4-34). Micro RNAs are small RNA molecules of 21 to 22 nucleotides, transcribed from cellular genes as long RNA precursors that are then cleaved by cellular nucleases in 21-22 nucleotide RNAs. MiRNAs can target messenger RNAs (mRNAs) by perfect or imperfect complementary binding, causing cleavage and degradation or translational repression of mRNAs (for review see 35).

miRNA-122 is a liver tissue-specific miRNA. It constitutes approximately 70% of all liver cellular miRNAs and it is expressed at high levels (around 66,000 copies per cell) (24). miRNA-122 has been detected in both human and mice livers, in cultured human hepatoma cell line Huh7 as well as in mouse Hepa 1-6 cells (4). HepG2 human liver-derived cell lines, on the other hand, are negative for the presence of miRNA-122 (4). miRNA122 function in the liver is related to lipid and cholesterol biosynthesis (5), in fact, inhibition of miRNA-122 by antisense RNA administration reduces fatty acid biosynthesis, plasma cholesterol, and triglyceride levels (22). Moreover, when miRNA-122 antisense was administered to mice with fatty livers amelioration in liver histology was demonstrated (36). Of interest, HCV replication is also related to lipid and cholesterol metabolism, since cholesterol biosynthetic products are considered critical for HCV replication (5). Since HCV is a positive-stranded RNA virus with a preferential tropism for hepatocytes, the role of miRNA-122 in modulating HCV replication has been further investigated. miRNA-122-HCV interaction is critical in maintaining viral abundance and ultimately HCV replication in Huh7 cells (4). On the other hand, HCV replicons are able to replicate in a variety of non-hepatocyte-derived cell lines such as HEK-293 (a human embryonic kidney epithelial cell line), mouse embryonic fibroblasts, and HeLa cells (a cervical cancer epithelial cell line) (15). Because these cell types do not express miRNA-122 (15, 37), these results indicate that miRNA-122 is not essential for HCV replication, at least in such non-hepatic cells. Of note, Chang *et al* have observed that exogenous expression of the liver-specific miRNA-122 in HEK-293 cells enhances the accumulation of HCV replicons RNA without affecting the stability of viral plus-strand RNA and its translation activity (15).

3. HOW DOES MiRNA AFFECT THE LIFE CYCLE OF HCV?

The mechanism by which the interaction of miRNA-122 with the HCV genome facilitates the increase in its abundance is only partially understood. miRNAs usually target mRNAs at the 3' noncoding regions, down-regulating mRNA expression (2,3). miRNA-122, on the other hand, binds to the 5' end of HCV leading to viral RNA abundance (Figure 1). The HCV 5' and 3'-untranslated regions, which flank the HCV open reading frame, contain four stem loops, stem-loop I and II in the 5'-UTR which are important for viral replication, while stem-loop III-IV contain the Internal Ribosome Entry Site (IRES) responsible for efficient cap independent translation of viral RNA (38,39).

Jopling *et al* identified two possible binding regions of miRNA-122, one in the 3' noncoding region (NCR) and a second sequence in the 5' NCR (4). Of importance, both sequences are conserved in different HCV genotypes. The authors have shown that miRNA-122 binds to the 5' NCR of HCV genomic RNA and is essential for HCV replication in human hepatoma-derived Huh7 cells (4). In a subsequent article, Jopling *et al* demonstrated a second adjacent biding site of miRNA-122, site 2 (Figure 1). Mutational studies further showed that both miRNA-122 binding sites are simultaneously occupied to maintain viral abundance (6).

In a more recent investigation, Jangra *et al* confirmed that miRNA-122 promotes replication of infectious virus by direct interaction with both seed sequence-binding sites in the 5' UTR (S1 and S2) (11). In this study, point mutations in both S1 and S2 viral sites impaired the production of infectious virus. In fact, viral production was rescued by miRNA-122 complementary mutations. However, substitutions in only one of the two sites revealed that binding to the 5'UTR S1 site is more important for efficient replication than binding to the nearby S2 site (11). Furthermore, miRNA-122 binding to both S1 and S2 sites, which are located upstream of the IRES in the 5' UTR, enhances HCV translation at the internal ribosome entry site (IRES) (11). These data are in accordance with Henke *et al* who reported that miRNA-122 upregulates HCV RNA translation at a ribosomal stage, demonstrating that sequestration of miRNA-122 by 2'-O-methylated anti-miRNA-122 oligonucleotides decreased HCV IRES-directed translation (14). These results are in contrast with the original study of Jopling *et al* which failed to demonstrate that miRNA-122 specifically enhances HCV IRES-directed translation (4).

4. MiRNA-122: CLINICAL CORRELATIONS

A recent article by Marquez *et al* analyzed the expression of miRNA-122 and miRNA-21 in liver samples obtained by liver biopsies of HCV-infected patients and uninfected controls (9). miRNA-21 was measured because of its suggested role in proliferation and tumorigenesis (40), while miRNA-122 levels are decreased in hepatocellular carcinoma and linked to cancer progression and acquisition of invasive properties (41-48). Marques' results indicate that miRNA-122 levels are inversely correlated with fibrotic stage, ALT and AST levels, and its expression decreased along with increased liver damage. On the contrary, miRNA-21 is positively correlated with

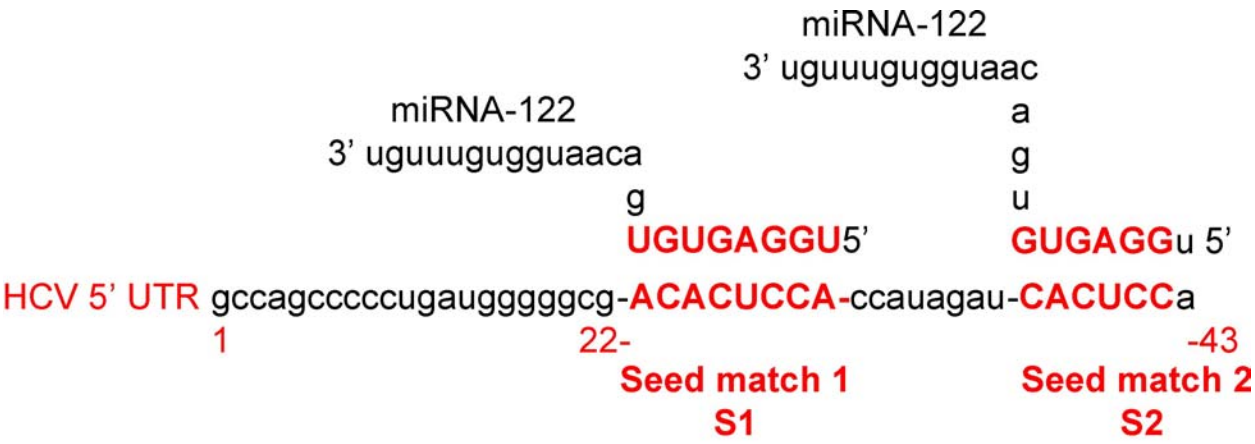

Figure 1. miRNA-122 binding sites, S1 and S2, in the 5' UTR of HCV RNA

fibrotic stage, viral load, ALT and AST levels. These findings were confirmed in tissue culture experiments where the authors found that HCV infection of Huh7 cells induced a parallel increase in miRNA-21 and a decrease in miRNA-122 at 144 hours post infection. The investigators speculated that the dysregulation of miRNA-21 and miRNA-122, rather than the expression levels, could be related with fibrosis and used the classical mouse model of liver fibrosis induced by CCL4 administration twice a week for 4 weeks to explore this possibility. miRNA-21 levels from CCL4 treated mice significantly correlated with fibrotic stage, while, miRNA-122 levels inversely correlated with fibrotic stage (9). Whether dysregulation of these miRNAs is the cause or the consequence of fibrosis remains to be further established. A study conducted by Filipowicz *et al* has also analyzed the levels of miRNA-122 in liver biopsies of 44 patients with chronic HCV infection undergoing IFN therapy (13). Interestingly, the authors found that patients who did not respond to IFN and ribavirin, with a decrease in viral load (primary nonresponders, PNRs), had lower pretreatment levels of miRNA-122 as compared with patients who responded well to therapy (complete early virological responders, EVR). Since IFN could modulate the expression of miRNA-122, miRNA-122 levels were measured before and 4 hours after INF treatment. However, no differences were noticed in either group before and after treatment and miRNA-122 levels did not correlate with viral load. Based on the fact that miRNA-122 is required for HCV replication and HCV viral loads were higher (although not significantly) in PNR group, the authors concluded that either the levels of miRNA measured in the liver samples do not reflect what really happens in HCV infected cells or that miRNA-122 is not a limiting factor (13).

5. INTERFERON BETA DOWN-REGULATES MiRNA-122

Pederesen *et al* have reported recently that IFN-Beta induces several miRNAs that can inhibit HCV RNA replication. More importantly, IFN-Beta treatment of Huh7 cells profoundly inhibited the expression of miRNA-122 (16). These experiments revealed that miRNA-196, miRNA-296, miRNA-351, miRNA-431 and miRNA-448 had almost perfect match with HCV RNA genome and they were all up-regulated by IFN-Beta. They further showed that these miRNAs were indeed capable of inhibiting HCV replication *in vitro*. Moreover, when Huh7 cells were treated with IFN-Beta a substantial down-regulation (80%) of miRNA-122 was demonstrated. These observations seem to suggest that miRNA-122 down-regulation by IFN-Beta could be a general antiviral mechanism, however, the other antiviral effects of interferon type I need to considered.

6. INHIBITION OF MiRNA-122 *IN VITRO*

Jopling *et al* analyzed whether miRNA-122 inactivation would regulate HCV gene expression (4). First they transfected Huh7 cells stably expressing the HCV genotype 1b with a 2'-methylated RNA oligonucleotide perfectly complementary with miRNA-122. HCV viral replicon RNA was reduced by 80% upon inactivation of miRNA-122. Testing RNA accumulation in the presence of a replication-competent HCV RNA led to similar results. To demonstrate the putative miRNA-122 binding sites responsible for RNA abundance, the authors introduced mutations in the 5' NCR of HCV genotype 1a H77c RNA. They showed that RNA with a single nucleotide mutation in the seed match of the 5' NCR of HCV failed to accumulate, suggesting that the interaction of miRNA-122 and the 5' NCR was critical for viral RNA abundance (4).

A recent study by Young *et al* reported the discovery of small molecule modifiers of miRNA-122, both inhibitors and activators. By using a reporter system, they showed that miRNA modifiers act at a transcriptional level (10).

7. INHIBITION OF MiRNA-122 *IN VIVO*

Preliminary experiments attempting to inhibit miRNA-122 *in vivo* have demonstrated long-lasting decrease of serum cholesterol in mice and African green monkeys using a locked nucleic acid (LNA)–modified phosphorothioate oligonucleotide (SPC3649)

complementary to the 5′ end of miRNA-122 (8, 22). SPC3649 has also been tested as a new anti-HCV approach in chronically infected chimpanzees (genotype 1) (12). Two animals were treated with the high-dose and two with low-dose (5 mg/kg and 1 mg/kg, respectively) with I.V. injections of SPC3649 on a weekly basis for 12 weeks followed by 17 weeks treatment-free period. In the high-dose group, a 2.6 and a 2.3 order of magnitude reduction in HCV RNA was detected in the serum and liver, respectively. miRNA-122 levels were measured by real-time reverse transcription polymerase chain reaction in liver biopsies of the high-dose animals revealing a factor of >300 decrease in free miRNA-122 levels. Moreover, no rebound in viraemia was observed during the 12-week dosing phase and deep sequencing of the HCV 5′ NCR showed no mutations in the miRNA-122 seed sites.

Safety of SPC3649 was assessed throughout the study by monitoring complete blood counts, blood chemistries, coagulation markers, urinalysis, complement activation, lymphocyte subsets, circulating cytokine-chemokine profiles, and other safety parameters. None of these parameters was altered at any time-point. Furthermore, the authors demonstrated improved liver histology in both high-dose animals, showing that prolonged suppression of viraemia and normalization of the IFN pathway also led to a histological response. The authors concluded that the efficacy of LAN oligonucleotides to suppress HCV RNA, along with histological response, its safety profile and the high-barrier to resistance, are encouraging data to build on new anti-HCV therapeutic strategies.

8. CONCLUSIONS

In vitro and *in vivo* studies suggest that anti-miRNA-122 SPC3649 could be a valuable drug to add to the HCV armamentaria. The LAN oligonucleotides SPC3649, which targets both total cholesterol in African green monkeys and HCV replication in the chimpanzee model have been demonstrated to be safe, effective and, in the HCV model, not to induce viral escape mutations. Phase I clinical trials, sponsored by Santaris Pharma, are already under investigation in adult healthy volunteers (5).

Some concerns regarding SPC3649 safety profile will certainly be taken into consideration. In fact, on one side, the effect of SPC3649 on lipid metabolism needs clinical vigilance to monitor cardiovascular events. On the other side, although SPC3649 inhibits HCV replication through a decrease of miRNA-122 contents, lower miRNA-122 levels have been detected in HCC (41-48).

Beside the safety profile, which is certainly critical, it will be of interest to monitor, *ex vivo*, whether viral mutations will emerge in HCV-infected individuals treated with SPC3649. If viral escape develops, a potent combination therapy may be needed. Moreover, the type of reciprocal effect of SPC3649 and interferon therapy also needs to be considered. Furthermore, the time to complete viral clearance and the possibility of viral reactivation upon treatment interruption will be of great importance for clinicians and scientists dealing with HCV infection.

Future investigations and clinical trials will reveal whether SPC3649 is going to be a possible therapeutic agent for HCV infection, alone or in combination with other anti HCV drugs.

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Send correspondence to: Giuseppe Nunnari, Department of Medicine and Medical Specialties, Division of Infectious Diseases, University of Catania, Via Palermo 636, Zip: 95123, Catania, Italy, Tel:39-095-7598443, Fax:39-095-7598666, E-mail: gnunnari@hotmail.com

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