REVIEW

New therapeutic opportunities for Hepatitis C based on small RNA

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Abstract

Hepatitis C virus (HCV) infection is one of the major causes of chronic liver disease, including cirrhosis and liver cancer and is therefore, the most common indication for liver transplantation. Conventional antiviral drugs such as pegylated interferon-alpha, taken in combination with ribavirin, represent a milestone in the therapy of this disease. However, due to different viral and host factors, clinical success can be achieved only in approximately half of patients, making urgent the requirement of exploiting alternative approaches for HCV therapy. Fortunately, recent advances in the understanding of HCV viral replication and host cell interactions have opened new possibilities for therapeutic intervention. The most recent technologies, such as small interference RNA mediated gene-silencing, anti-sense oligonucleotides (ASO), or viral vector based gene delivery systems, have paved the way to develop novel therapeutic modalities for HCV. In this review, we outline the application of these technologies in the context of HCV therapy. In particular, we will focus on the newly defined role of cellular microRNA (miR-122) in viral replication and discuss its potential for HCV molecular therapy.

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INTRODUCTION

Hepatitis C virus (HCV), first identified in 1989, is a single-stranded positive-sense RNA flavivirus with 6 major genotypes and over 70 subtypes[1,2]. According to the estimation of the World Health Organization, approximately 170 million people, 3% of the world population, are HCV positive with 3 to 4 million de novo infections each year. Unfortunately, 55%-85% of those infected fail to clear the virus and progress to develop chronic infection. Over a period of 20 to 30 years cirrhosis develops in about 10% to 20% and hepatocellular carcinoma (HCC) develops in 1% to 7% of persons with chronic infection[3]. Currently, no safe and effective vaccine is available to prevent HCV infection. Conventional treatment, such as interferon taken alone or in combination with ribavirin, is only effective in part of the patients, but is often financially inaccessible for people in developing countries[4,5].

To explore the potential of new therapeutic strategies, it is critical to better understand the viral and host factors involved in virus cell entry, replication and virus-cell interaction. An apparent two-way dialogue exists in which the virus apparently takes advantage of the cells' own signal transduction systems to facilitate virus entry and support replication[6]. Indeed, remarkable progress has been achieved in understanding the properties of the HCV genome and viral proteins. Contributions have come through several different sources, including vaccination of chimpanzees, structural studies, binding studies with recombinant envelope proteins, and the use of clinical isolates, HCV-like particles (HCV-LPs), HCV pseudotyped particles (HCVpp), and cell culture-derived HCV particles (HCVcc) in infectivity assays[7,8]. Cellular pathways or molecules involved in viral entry, such as CD81, scavenger receptor class B type I (SR-BI), LDL receptor, L-SIGN, DC-SIGN and asialoglycoprotein receptor (ASGPR) could be putative therapeutic targets[9-12].

New technologies, particularly RNA interference (RNAi) induced by small interfering RNA (siRNA), are gaining favour as effective therapeutic entities for HCV infections. RNAi works at a posttranscriptional level by

degrading cognate mRNA. As HCV is a single-stranded RNA that functions as both a messenger RNA and a template for replication, it is a prime candidate for RNAi. Moreover, previous reports have shown that by blocking cellular determinants of viral entry and replication, such as CD81, HSP90, or p68, either by RNAi, antisense oligonucleotides or chemically engineered "antagomirs", leads to significant reduction of viral invasion[13-15]. In this review, we outline the novel small RNA based technologies in designing therapeutic approaches for HCV treatment, according to the mechanism of viral entry, replication and virus-cell interaction. In particular, we will discuss emerging evidence that a liver-specific, small non-coding microRNA (miRNA) is involved in replication of HCV through a novel mechanism and outline its therapeutic potential.

MOLECULAR CHARACTERISTICS OF HCV ENTRY AND REPLICATION

HCV, contains a single-stranded RNA genome of about 9400 nucleotides in length, composed of a 5' and 3' non-coding region (NCR) with a single open reading frame encoding a polyprotein precursor of approximately 3000 amino acids that is cleaved into three structural (core, E1, E2) and seven non-structural (p7, NS2-NS5B) proteins[16,17].

Since the discovery of HCV, numerous studies have demonstrated its mechanism of cell entry, but it is still unclear how the virus penetrates cell membranes. In order to elucidate the infection pathway, it is first required to identify and understand both the putative viral and cell factors involved in this process. The viral envelope glycoproteins E1 and E2, cleaved from the polyprotein by the endoplasmic reticulum (ER)-resident host enzymes signal peptidase and signal peptide peptidase, have been widely regarded as the critical determinants for virus cell entry. To date, several models have been designed to investigate E1/E2 function. These include HCV-LPs expressing E1-E2 heterodimers instead of glycosylated individual E1 and E2[18-21], HCVpp consisting of unmodified HCV envelope glycoproteins E1 and E2 assembled onto retroviral or lentiviral core particles[22-26], vesicular stomatitis virus (VSV)/HCV pseudotypes expressing HCV E1 or E2 chimeric proteins containing transmembrane and cytoplasmic domains of the VSV G glycoprotein, or HCVcc neutralization assays with E1 or E2 antibody[27-30]. These models have shown that both envelope glycoproteins E1 and E2 are essential for host cell entry. The lack of either E1 or E2 significantly decreases HCV infection activity whereas deletion of the whole envelope protein coding sequence abolishes the particle infectivity. Additionally, several cell surface molecules have been identified using these models and are now considered as critical components in mediating HCV attachment and entry.

Similar to viral entry, HCV replication requires both viral and cellular factors. Although our current knowledge of the HCV life cycle is still mainly at the hypothetical level, several minimum viral components and host cell factors have been proposed. The HCV 5' NCR, in

particular the IRES sequence, plays an important function in ribosomal assembly and the NS3 to NS5B coding region are necessary for function of the replicase complex[31-35]. Found as interaction partners of NS5A and NS5B, human vesicle-associated membrane protein-associated proteins VAP-A and VAP-B were first identified from the host cell[36,37]. More recently, the geranylgeranylated protein FBL-2, the immunophilins cyclophilin B and FKBP8 have been identified as important host factors for HCV replication[38-40]. Furthermore, the host enzyme IMPDH, essential for the *de novo* synthesis of GTP nucleotides, may be involved in HCV replication as the IMPDH inhibitors ribavirin and mycophenolic acid suppresses replication[41,42]. Interestingly, the mammalian liver-specific miRNA (miR-122) has been recently defined to facilitate HCV replication, indicating that this small RNA may present a novel target for antiviral intervention[43].

miR-122 AND HCV REPLICATION

miRNAs are approximately 22 nucleotide noncoding RNAs that can downregulate various gene products by inducing either cleavage or a reduction in the translational efficiency of the target mRNA[44,45]. In the last 5 years, over 3000 miRNAs have been identified in vertebrates, flies, worms, plants and even viruses. Most miRNAs have been shown to participate in essential biological processes, such as cell proliferation, apoptosis, differentiation and metabolism[46]. The 22 nucleotide mature miR-122, derived from a noncoding polyadenylated RNA transcript of the hcr gene, is a liver-specific developmental regulator. It can be detected as early as 12.5 d post-gestation and reach a plateau immediately before birth, then slowly increase up to 70% of the total miRNA population in adult liver[47-49]. miR-122 is the first identified host miRNA linked to HCV viral replication. A further novelty to these findings is the fact that miR-122 upregulates, rather than downregulates, viral RNA by interaction with the 5' NCR of the viral RNA. Previous work had suggested that miRNA can only negatively regulate gene expression through targeting the 3' NCR of mRNA.

Interestingly, Jopling *et al*[43] have observed that though both Huh7 and HepG2 cells are derived from human hepatocytes, HCV RNA can only replicate in Huh7 cells. This may link to the fact that Huh7 is miR-122 positive, while HepG2 is miR-122 negative. To determine if miR-122 is required to regulate HCV replication, they transfected antisense oligonucleotides into Huh7 liver cells to suppress miR-122 function. The results showed that the amount of viral RNA was reduced by about 80% when miR-122 was silenced, but it is still unclear whether it is simply a direct or indirect interaction through cellular factors. Thus, to further address this issue, two putative binding sites, located in each of the viral NCR, were tested as possible targets for miR-122. It was found that only the binding sequence located in the 5' NCR was responsible for miR-122 targeting. This is notably very different from the common observation that miRNA target the 3' NCR, leading to suppression or degradation of target mRNA. Recently, a study in mice has shown synthesized antisense single-stranded 23-nucleotide RNA molecules

can effectively inhibit production of miR-122 *in vivo*[50]. Therefore, miR-122 seems a potential target for HCV treatment, although the mechanism for this new miRNA role is still very much unclear.

THERAPEUTIC STRATEGIES BASED ON GENE SILENCING TECHNOLOGY

As current antiviral regimens have proven largely unsatisfactory, particularly for patients with genotype 1 infection, it is important to explore novel therapeutic strategies. Small interfering RNAs and antisense oligonucleotides (ASO) have emerged as efficient nuclei acid-based gene silencing tools to target highly conserved or functionally important regions within the HCV genome or essential host cell factors for entry or replication (Figure 1).

RNAi, induces gene silencing at a post-transcription level by double-stranded small interference RNA (siRNA) and represents an exciting new technology that could have applications in the treatment of viral diseases. Particularly, HCV could be an attractive target for RNAi therapy, as it is a RNA virus. The HCV genome is a positive single-stranded RNA that functions both as the viral messenger RNA and a template for RNA replication *via* a negative-strand intermediate. Instead of a 5' cap, the IRES, located at the 5' NCR, plays an essential role to bind eukaryotic ribosomal subunits and initiates the assembly of the translationally active 80S complex. Consequently, this sequence is more conserved than any other part of the viral genome, at least among the six known HCV genotypes[51,52]. Thus, IRES seems an ideal target for RNAi mediated anti-HCV therapy and several groups have demonstrated efficient inhibition of HCV replication by designing siRNAs toward this region[53-55]. In addition, RNAi directed against the viral core, NS3, NS4B, NS5A and NS5B regions can suppress HCV infection. McCaffrey *et al*[56] was the first to demonstrate feasibility of siRNA targeting HCV NS5B *in vivo*. By co-expression of an NS5B-luciferase fusion gene with an anti-NS5B siRNA expression plasmid they found a significant reduction of luciferase expression in the mouse liver indicating selective degradation by the NS5B siRNA. Additionally, several other groups have observed suppression of HCV replicon by siRNA-mediated targeting either NS5B or NS3 region[57-59].

Besides these viral elements, numerous host cellular factors, such as CD81, SR-BI, HSP90, p68 or USP18, could be typical targets for potentiating RNAi antiviral therapy. CD81, expressed in most human cells, is able to bind to HCV E2 protein and is, therefore, considered an essential receptor for HCV entry. Further investigation, by either ectopic expression of CD81 in Huh7-Lunet cells (low expression of CD81) or modulation of CD81 cell surface density in Huh-7.5 cells (high expression of CD81) by RNAi, revealed that density of cell surface-exposed CD81 is a key determinant for HCV entry into host cells[60]. SR-BI, primarily expressed in the liver and steroidogenic tissues, was identified as another potential HCV receptor based on coprecipitation with recombinant E2. A 90% down-regulation of SR-BI expression in Huh7 cells by
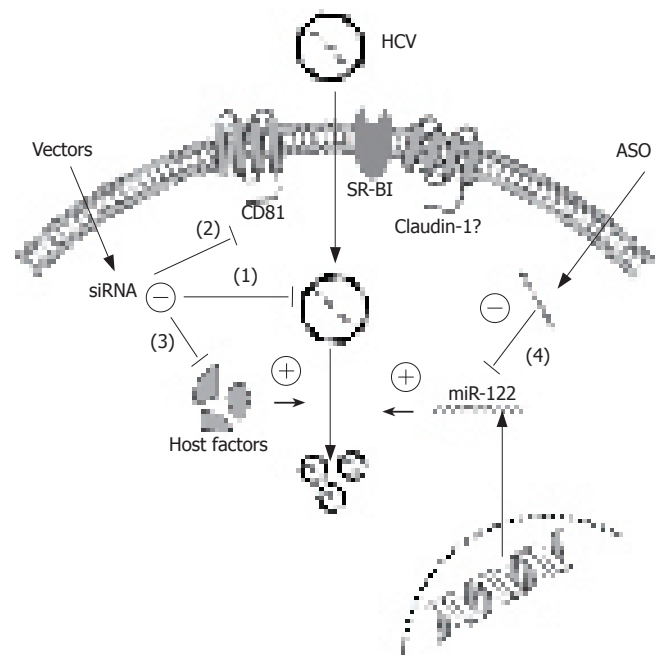

Figure 1 Novel anti-viral strategies based HCV life cycle. RNAi technology, inducing gene silencing at posttranscriptional level mediated by siRNA, can be applied to prevent HCV replication and infection by targeting either viral RNA (1) or host factors, such as surface receptor (2) or cellular molecules (3). miR-122 is a liver specific miRNA that is involved in HCV replication and therefore silencing of miR-122 by antisense oligonucleotides (ASO) could be considered as a potential therapeutic modality (4).

RNAi caused a 30%-90% inhibition of HCVpp infection, depending on the HCV genotype[61,62]. However, either CD81 or SR-BI alone is not capable of virus binding indicating that at least one additional host protein, possibly the recently identified co-receptor, Claudin-1[63], is required for cell entry of enveloped virions *via* the CD81/SR-BI pathways.

Although using siRNA to target either viral or host factors could be considered effective tools to significantly block HCV infection and replication, an advanced method by knockdown both viral and cellular factors may further improve the therapeutic efficacy. Work by our group has shown that both entry and replication can be simultaneously targeted using shRNAs directed against two regions of the HCV RNA and one region of the host cell receptor, CD81. The triple shRNA expression vector was effective in concurrently reducing HCV replication, CD81 expression, and E2 binding, comparable to conventional single shRNA anti-HCV vectors[64].

Antisense oligonucleotides represent an alternative gene-silencing tool that can be employed as HCV therapy. ASO-based inhibition of HCV has been demonstrated extensively in the past[65-71]. Currently, ASO is the most promising method to block the function of miRNA, such as miR-122. For instance, a 2'-O-methylated RNA oligonucleotide with exact complementarity to miR-122 was introduced to inactivate its function in Huh7 cells, in order to determine the relationship between miR-122 and HCV replication. Subsequently, Krutzfeldt *et al*[50] developed a pharmacological approach for silencing miRNA *in vivo*, by chemically modified,

cholesterol-conjugated single-stranded RNA analogues to complementarily target miR-122. By injection of these 'antagomirs' into the tail veins of mice, efficient and specific suppression of endogenous miR-122 was observed. Hence, designing ASO based molecular medicines would provide new agents for human major diseases, because upregulation of certain miRNAs linked to a set of diseases such as cancer, diabetes or HCV.

LIVER-TARGETED VIRAL DELIVERY SYSTEMS

Obviously, RNAi or ASO technologies could be regarded as potentially effective novel modalities for anti-HCV treatment. Nevertheless, the success depends on developing effective delivery systems, to target therapy to the liver. Regarding to treat a liver-hosted and long-term persistent hepatitis virus, an ideal vector would be able to transfer genetic material efficiently and specifically into the target cells/tissues, resulting in high level, properly regulated and prolonged expression, without toxic and immunogenic side effects. Since viruses have many advantages as transgenic vehicles, we will discuss two of the most promising delivery systems: lentiviral and adeno-associated viral (AAV) vectors.

Lentiviral vectors, are mainly based on human immunodeficiency virus type 1 (HIV-1) and have been shown to effectively transduce liver, muscle, and hematopoietic cells. These vectors integrate their payloads into the host genome ensuring transmission to progeny cells[72]. Although lentiviral-mediated short hairpin RNA (shRNA, precursor of siRNA) delivery has been widely developed for therapeutic application, there are few reports referring to HCV treatment[57,64]. There are currently some limitations for the use of lentiviral vectors: (1) production efficiency limits *in vivo* transfection; (2) possibility of insertional mutagenesis or generation of wild-type virus leading to safety considerations. To circumvent these drawbacks the following strategies may be required to achieve further improvement: firstly, newer generations, such as the gutted third generation, relatively high titers of VSV-G pseudotyped HIV-1 vectors, other types such as HIV-2 and simian immunodeficiency virus (SIV) vectors, or even immunodeficiency viruses derived from nonprimates, including felines and equines, are also being developed to overcome conventional problems[73-76].

Analogically, with the superiority of low pathogenicity and long-term gene expression, AAV could be another ideal viral vector for siRNA delivery, although no reference of AAV-mediated anti-HCV RNAi therapy has been reported so far. Particularly AAV serotype 8, a new member of the AAV family isolated from rhesus monkeys, is an attractive candidate for hepatic-directed shRNA transfer because of 10- to 100-fold increased transduction efficiency in mouse liver models, compared with the previous AAV2 based vectors[77]. Since derived from nonhuman primate, AAV8 is less prone to recognition by prevailing antibodies that generate side immunological effects in human[78]. Moreover, the safety and transgenic delivery efficacy could be further improved by conjugating other strategies, such as utilizing liver-specific promoters, hybridization of AAV8 with other serotypes, or modification of viral capsids.

Furthermore, since miRNA context based siRNA cassette (second-generation shRNA) can be driven by a regulated pol II promoter instead of conventional pol III promoters[79], liver-targeted expression of shRNA could be achieved by employing a liver-specific pol II promoter in viral delivery system.

CONCLUSION

The treatment of HCV remains a challenge that requires further elucidating the process of viral life cycle and developing novel therapeutic approaches. In fact, recent progress has provided the possibilities of identifying novel antiviral targets and designing new therapeutic strategies. According to the previous description, miR-122 is one of the most emergent targets for HCV therapy that is commonly abundant in human livers and thus promotes viral replication. Therefore, downregulation of miR-122 by antisense based 'antagomirs' or oligonucleotides significantly suppressed viral replication. However, before such a method can be applied in the clinic, the role of miR-122 in maintaining normal hepatic function must be further investigated. Krutzfeldt *et al*[50] have demonstrated that silencing of miR-122 by 'antagomirs' do not show any apparent toxicity to mice, but the more recent study has shown that miR-122 is downregulated in the rodent and human hepatocellular carcinomas (HCC). Using the animal model of diet-induced hepatocarcinogenesis, Kutay *et al*[80] have observed that the reduced expression of miR-122 probably occurs between 36 and 54 wk when neoplastic transformation occurs. These findings suggest that the downregulation of miR-122 might be associated with hepatocarcinogenesis and, therefore, further investigation into the function of miR-122 is required before therapeutic application can be commenced. In conclusion, the recent progress of understanding the viral life cycle and identification of novel targets, in combination with the newly developed ASO and RNAi technology, may pave the way for new anti-HCV therapy.

ACKNOWLEDGMENTS

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