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REVIEW ARTICLE

Role of circulatory microRNAs in the pathogenesis of hepatitis C virus

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Abstract Hepatitis C virus (HCV) is associated with one of the major health problem in world that ultimate results in the liver cirrhosis and leads to carcinoma of hepatocellular components round the world. More than 185 million people were found to be infected with HCV. MicroRNAs are small oligonucleotide RNA having 18–22 nucleotides. Circulating mi-RNAs regulate the replication of HCV and HCV-induced liver fibrosis and HCC. By comparing the expression profiles of mi-RNAs of normal individuals with HCV infected patients, aberrant changes in expression of different mi-RNAs have been observed so it can be predicted that these mi-RNAs are associated with and play a central role in the hepatitis C infection and diseases associated with it. This review demonstrates the major role of circulatory microRNAs in the HCV and HCV associated ailments.

Keywords HCV · HCC · MiRNA · Pathogenecity

Introduction

Hepatitis C virus (HCV) is a icosahedral hepatotrophic virus having a small size of 55–66 nm, belonging to virus family Flaviviridae [38]. The genome size is approximately 9.6 KB encoding 10 proteins including two envelop proteins E1 and E2, core protein, and seven nonstructural proteins NS1, NS2, NS3, NS4a, NS4b, NS5a and NS5b [8].

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Hepatitis C virus is associated with one of the major health problem in world that ultimate results in the liver cirrhosis and leads to carcinoma of hepatocellular components round the world. More than 185 million people were found to be infected with HCV [45]. It infects approximately 3% of the world's population and it has been examined in 30% of infected people that develop end stage liver disease eventually [3]. In 2004, according to the World Health Organization (WHO), about 0.3 million and 0.8 million deaths are estimated annually due to HCV and liver cirrhosis respectively. In 2010, it was found that 10 million people are infected by HCV in Pakistan [1].

The genome of HCV is flanked by 5′ and 3′ un-translated regions (UTRs) that are needed for replication and to initiate translation. The 5′ UTR consists of an extensive secondary structure like an internal ribosome entry site (IRES) that mediates translation, binds ribosomal protein S9 and eIF3 [9]. Moreover, in the 5′ UTR other sequences are required for replication of the negative strand. The 3′ UTR also consists of a long secondary structure that is responsible for replication of genome. There is verification for RNA–RNA interactions between the 5′ and 3′ UTRs and also between RNA sequences at the C-terminus of NS5B and 5′ UTR. These bonding are necessary for replication and boost translation strongly from the HCV IRES [62]. The 5′ and 3′ UTR's are also needed for encapsulation as both of them interact with the core protein [79]. After translation on the rough endoplasmic reticulum (RER), the poly-protein precursor is digested into 10 proteins by both host and viral proteases. These proteins form an association with the endoplasmic reticulum and cellular membranes are modified to produce the membranous web on which viral replication takes place [32]. Maturation and assembly of virus occurs in association with lipid droplets. Host signal peptidase processes the three structural

proteins, core, Envelope 1, Envelope 2, and the viral porin p7. Processing of core protein yields mature form that has the ability to associate with lipid droplets. The viral capsid formed by the core protein interacts with both the 5′ and 3′ UTRs, and also with the E1. The interaction depends upon oligomerization of core protein. Both E1 and E2 form heterodimers on the Endoplasmic reticulum and then their glycosylation occurs but are not further modified by Golgi enzymes showing that they retain in the Endoplasmic reticulum [32].

Till now, seven different genotypes of HCV have been identified on the basis of their whole genome sequence analysis and phylogenetics. These subtypes are further classified into 67 subtypes [53]. The cause of this variation is the high mutation rate of its error prone RNA polymerase. The prevalence of HCV has caused it to achieve the status of an epidemic. Population in Pakistan is majorly effected by HCV genotype 3a with few cases of genotype 1a. In developing countries like Pakistan, HCV prevalence is growing mainly due to lack of fulfillment of international standards regarding poor sterilization practice [1].

Enzyme immunoassay (EIA) is an assay to detect hepatitis C antibody, whereas the viral load are measured by hepatitis C RNA assays. Genotype 1 and 3 are more prevalent, although 3a is the most abundant genotype in Pakistan. Interferon therapy has shown better treatment results for HCV genotype 2 and 3, whereas, the HCV Genotype 1 infection has some extent of resistance to treatment [51]. Direct acting antivirals (DAAs) are orally taken and free from interferon, inhibit the viral replication by targeting the non-structural proteins of virus. DAAs like Boceprevir and telaprevir are NS3/4A protease inhibitors, have shown better sustained virological response (SVR) results as compared to the interferon therapy [13, 52].

Mi-RNAs and cellular non-coding RNAs

Approximately 97% of human genome is considered as the non-protein coding genome. So, it is sometimes called as "junk DNA" [14, 22]. Recently, research efforts have been made to identify the role of non-coding RNA. The non-coding RNA can be classified into structural and regulatory RNAs. The regulatory non-coding RNAs are more dividing into long non-coding RNAs and others siRNA, piRNA and microRNA (MiRNA) [21, 43, 54, 61].

MicroRNAs are small oligonucleotide RNA having 18–22 nucleotides. Lin-4 mi-RNA was identified as a first microRNA in *Caenorhabditis elegans* in 1993 [33, 75]. Let-7 mi-RNA was the 2nd identified microRNA that was extremely preserved in both vertebrates and invertebrates [49, 55]. MicroRNA is the major class of non-coding RNA that controls the expression of the protein thus considered as a post transcriptional regulator of gene expression. It either cleaves the mRNA or suppresses the translation by binding to mRNA.

Some viruses have mi-RNAs encoded genes and most of the viruses interact with host cellular and circulating mi-RNAs during their life cycle. HCV interact with cellular mi-RNAs to regulate their life cycle by binding with messenger RNA (mRNA) to degrade it or repress its expression after post-transcription [19]. Mi-RNAs show unusual expression and role in different human diseases [20, 44]. In HCV-liver associated diseases including liver fibrosis and cellular carcinoma, mi-RNAs have an aberrant expression [74]. Therefore, molecular mimics and antagonists of specific mi-RNAs use for modulation of cellular mi-RNA expression can play a promising role in therapeutics during HCV infection.

Mi-RNA biogenesis and activity

Mi-RNA plays an important role in controlling the expression of genes. Mi-RNAs are actually non-coding RNA and their biogenesis mainly occurs in the nucleus by the formation of primary mi-RNA transcript (pri-MiRNAs) during transcription by RNA polymerase II. Like mRNA, pri-MiRNAs are also capped and have poly-adenylated tail, but they are not encoded RNA. Hairpin like structure is formed due to one or more poorly complementary regions present in the pri-MiRNAs which is later processed by a series of ribonuclease cleavage events into mature mi-RNA. First of all, in the nucleus, pri-MiRNAs is cleaved into individual hairpins called pre-MiRNAs by Drosha, RNaseIII enzyme and DGCR8, RNA binding protein [29, 68]. Then, Exportin-5, a transport protein, transports the pre-MiRNAs from the nucleus to the cytoplasm. Dicer with RNAse III enzyme and TRBP2, the double-stranded RNA binding protein cleave the hairpin lop and produce a mature mi-RNA. Later then, during its processing, mature mi-RNA are captured by RNA-induced silencing complex (RISC), where they directly attach to Argonaute (Ago) protein present in core of the RISC. Human have four Ago homologues, Ago 2 have the cleavage activity among them and is the only enzyme involved in si-RNA mediated knockdown [68]. Following binding with RISC, the mature mi-RNA duplex is unwounded, leaving the guide strand in its intact form and degrade the passenger strand [7, 28]. Ago 2 enzyme present the guide strand (single stranded) mi-RNA with the conformation that it binds with its complementary mRNA [57]. The target mRNA is cleaved in si-RNA mediated knockdown by Ago2 if the RISC binds with target mRNAs by perfect complementarity. If the imperfect complementarity between RISC and the target mRNA occurs, then mRNA translation silencing and then degradation occur. Till now, more than 2500 human MiRNAs have been recognized (MiRBase version 21) [77].

Moreover, a number of mi-RNAs can regulate each mRNA and involve in expression control. Therefore, it can be considered that MiRNAs form a complex gene regulatory framework that controls the every cellular function including cell division, cell growth, differentiation of cell and metabolism. Recent work on mi-RNAs focus on the identification of role of specific mi-RNAs in different diseases by analyzing the alteration in their expression profile using different methods such as microarray, sequencing method or q-PCR. Bioinformatics methods including computational analysis identify the potential target mi-RNA, but experiment verification requires for the confirmation. Computational commands are increased by the discovery of 'seed sequence' which is most important and effective in the suppression of target mRNA [36]. As the knowledge about mi-RNA regulated disease framework is unknown, so the key mi-RNAs having a role in pathogenesis of HCV have to be recognized.

Mi-RNAs that modulate the HCV life cycle

Mi-RNAs play an important role in the regulation of biological process of cells that occur in body, so it can easily be predicted that cellular and circulating mi-RNAs regulate the replication of HCV and HCV-induced liver fibrosis and HCC. 70% of population of mi-RNA in liver tissues is MiR-122 [48]. By comparing the expression profiles of mi-RNAs of normal individuals with HCV infected patients, aberrant changes in expression of different mi-RNAs have been observed so it can be predicted that these mi-RNAs are associated with and play a central role in the hepatitis C infection and disease associated with it [67]. The main target of mi-RNAs and their regulation are shown in Table 1. These mi-RNAs can be used as possible therapeutic objectives to control and treat the hepatitis C infection and disease associated with it.

MiR-122

The relation between mi-RNAs and the HCV infection was first identified with the discovery of mi-RNA MiR-122, most abundant mi-RNA in liver, plays a central role in the accumulation of HCV RNA in the infected tissue using an uncommon method [25, 26]. MiR-122 stabilizes the genome of HCV and also stimulates the HCV translation by annealing at the two adjacent sites of 5′ UTR of the HCV genome [24, 26].

MiR-122 have a seed sequence at 5′ UTR region at position 2–8 nucleotide complementarity with the target mRNA that is responsible for the suppression of translation of target mRNA. But the accumulation of HCV in infected cells requires annealing of 5′ seed nucleotide sequence and 3′ nucleotide sequences of mi-RNA. MiR-122 binding to the target, specially the binding of 3′ nucleotides produces 3′ MiR-122 overhang generates a hypothesis that it stabilizes the HCV RNA from host nucleases or from identifying by antiviral restriction elements of host by masking the 5′ end of the HCV genome [41] and thus provide a data in support of HCV RNA stabilization [37]. MiR-122 does not have a direct role in the HCV RNA polymerization [25, 73] but its role in the regulation of HCV replication has not been excluded. A study revealed that HCV genome conformation generated among 5′ UTR sequence and core coding region sequences can be modified by the MiR-122 [16].

However, the role of MiR-122 mechanism in regulation of HCV is quiet unidentified, but some of the proteins involved in the host mi-RNA pathways are essential. Canonical biogenesis of pre-MiR-122 into mature MiR-122 is necessary for its proper function. Canonical biogenesis includes the cleavage of pre MiR-122 by dicer and TRBP, RNA binding protein, into mature MiR-122 [81]. MiR-122 gene, located on chromosome 18, is transcribed into approximately 4.5 KB pri-MiRNA by polymerase II which is further processed into pre-MiRNA comprises of 66 nucleotides and become mature in cytoplasm. Different transcriptional factors enriched in liver have been responsible for MiR-122 activation that includes C/EBPα, HNF1α, HNF3β, and HNF4α and HNF6. Core promoter region has been identified from where transcription starts. MiR-122 belongs to poly A-RNA group, where the stem loop of pre-MiRNA act as a terminator instead of polyadenylation of tail [66]. For the activity of MiR-122 Ago2 with other Ago proteins is required, as the synthetic mimic of MiR-122 does not affect the HCV replication so it can be predicted that MiR-122 is selected by Ago2 protein and the delivery of guide strand to the HCV genome is also regulated by Ago2. Different studies fail to resolve that either Ago2 is required for the further steps of mechanism involved for MiR-122 activity [76, 81]. Ago2 protein is transported in the complex formed during HCV infection thus it indicates that Ago2 may have a role in the HCV replication [4]. The proper identification of role of Ago2 protein and MiR-122 in HCV infected patients requires a lot of work.

MiR-122 is most abundant mi-RNA present in liver, constituting almost 70% of mi-RNA population [35]. Cholesterol biosynthesis is reduced by the down regulation of MiR122 as the oxidation of fatty acids increases as a result [18, 46]. In an experiment on mice, increase in the level of MiR-122 increases the level of cholesterol so it can be hypothesized that MiR-122 targets the gene for proteins involved in the cholesterol biosynthesis [30, 69]. Mice with MiR-122 knockdown, shows the activation of the oncogenic pathways and the production of inflammatory products in the cells, indicating that MiR-122 have an anti-

Table 1 microRNAs, their target and roles in HCV propagation and replication

microRNAs	Target	Diseases associated with mi-RNA	Expression during disease propagation
microRNAs that regulates HCV replication			
miR-122	HCV IRES, Xrn1, Cyclin G1, SOCS3	HCV	Upregulation in HCV
miR-141	DLC1	HCV	Upregulated
miR-130	IFITM1	HCV	Upregulated
miR-21	MyD88	HCV	Upregulated
	IRKA1		
microRNAs that inhibit HCV replication			
miR-196	HCV NS5A	HCV	Downregulated
	Bach1		
miR-1	Unknown	HCV	Downregulated
miR-198	Unknown	HCC	Downregulated
Let-7a	NS5B and 5′ UTR sequences	HCV	Downregulated
Let-7b	IGF2BP, HCV NS5A, HCV IRES	HCV	Down regulated
miR-448	HCV Core Region	HCV	Unknown
miR-199	5′ UTR	HCV	Unknown
MicroRNAs role in fibrosis and Hepatocellular carcinoma (HCC)			
Let-7e	TLR4	HCV fibrosis	Upregulated
Let-7g	C-myc, p16INK4A, COL1A2	HCC	Downregulated
miR-200c	FAP1	Fibrosis	Upregulated
miR-449a	NOTCH1	Liver inflammation and fibrosis	Downregulated
miR-221/222	CDKN1B	Fibrosis	Upregulated
miR-21	SAMD7	Fibrosis	Upregulated
miR-155	APC	HCC	Upregulated
miR-29	COL1A1, COL3A1	HCC	Downregulated
miR-199	PAK4, c-Met	HCC	Downregulated

inflammatory and anti-tumorigenic activity [23]. High expression of miR-122 has been observed in HCV infected HCC and CHC Egyptians' patients with genotype 4 that indicated its major role as a biomarker for HCC followed bymiR-21 [15]. Mimics of MiR-122 as a therapeutic reagent for the treatment of HCV can be proved helpful but care should be taken.

MiR-198

MiR-198 is considered a s a modulator of cyclin T [63] and in the huh7 cells, involved in controlling expression of the HGF receptor, Met [65]. MiR 198 has a role in the down regulation of HCC. It has been observed that MiR-198 shows down regulation up to fivefold in HCC patients as compared to the normal control. Gradual decrease in MiR-198 is observed from cirrhosis to HCC, depending on the degree of severity. Main role of MiR-198 is tumor repression in carcinogenesis. Gene for MiR-198 is located at the untranslated 3′ region of follistatin, an active binding protein, and have unknown target mRNA that are suppressed by it [70]. In non-alcoholic fatty liver disease (NAFLD), the down regulation of MiR-198 has also been observed [11]. Up regulation of the MiR-198 in hepatoma cells leads toward the inhibition of cell migration and growth of cells, so MiR-198 acts as a tumor suppressor by repression of mitogenic and motogenic pathways diminishing cell growth and migration [17].

MiR-1

Change in expression of mi-RNAs have been observed in the cells of immune system in Chronic HCV infection patients who failed to eliminate viruses, that become a source of dysfunctional immune system [5, 60, 72].

Effect of interferon β as an antiviral agent against HCV in the peripheral blood mononuclear cells (PBMCs) changes the expression of mi-RNAs profile in chronic hepatitis C patients. The level of MiR-1 has been observed in the cells that are induced by interferon β. Increase in MiR-1 indicates that it targets HCV genome [58].

MiR-1 have a key role in the up-regulation of hepatitis B virus (HBV) as it has been observed that the replication of HBV showed a noticeable increase when the hepatoma

cells are exposed to the mimic of MiR-1. MiR-1 also enhances the expression of antigen and release of HBV in hepatoma cell line of HBV by regulating the host genes [80].

Let-7 family

A couple of studies on the role of mi-RNAs have shown gene expression can be regulated by binding at 5′-UTRs. Let-7 binds to the multiple sites present in the upstream region of internal ribosome entry site (IRES) of HCV in the 5′-UTR of a mRNA and suppress the protein synthesis of the target mRNA [40].

Lethal 7 (let-7) gene is one of the earlier discovered mi-RNA and was first discovered as an important gene of development in *C. elegans*) [55]. By using bioinformatics, BLAST, the same sequence of nucleotide of mature let-7 had been revealed in human and *Drosophila melanogaster* genome [49, 50]. Sequence of mature let 7 is highly reserved in animals [31, 34]. Members of let 7 family includes let 7a, let 7b, let 7c, let 7d, let e, let 7f, let 7g, let 7i, let 7j, let 7k, MiR-98 and MiR-202 [56].

It is considered as of the first identified mi-RNA in human [49] that is actually associated with different mechanisms such as under expression of cancer and cellular differentiation [64, 78]. Let 7b is a negative regulator of HCV having a role in the control of HCV pathogenesis. So, let 7b have an antiviral role. It is also stated that let 7b has the same effect on different genotypes of HCV. There is a direct interaction between the let 7b and HCV genome.

Increase in Let-7a expression reduces the replication of HCV in HCV replicon as well as reduction in viral load has been observed in HCV infected cells. By using bioinformatics tools, it was predicated that let-7a, let-7c, and let 7f were also liver abundant mi-RNAs. The possible mi-RNAs roles were tested in HCV cell lines. Let-7a was under expressed whereas let-7c was overexpressed in the HCV cell line [10]. This study indicated strong effect of let-7a in the inhibition of HCV whereas our data shows that let-7a helps the HCV in its replication and stability. By computational analysis, NS5B and 5′ UTR sequences were the target sites for let-7a as these sites are conserved between different virus genotypes [10, 67]. In other studies, increase in the level of let-7a and let-7c has been observed in HCV associated tumor and non-tumor tissues, increase of let-7a-2 in HCC and liver fibrosis tissues [27, 71]. In HCV associated liver fibrosis, Let 7 family revealed a strong association. Expression levels of Let-7a, let-7c and let-7d reduced in early liver fibrosis stage [42].

Up-regulation of Let 7i have a role in the HCC has been published in a study of comparison of expression profile of mi-RNAs in 40 HCC patients and 6 non-tumor tissues [27]. In HCC, let-7 family had an anti- tumor activity as it suppresses the HCC. By down regulating the BCL-xl protein that decreases the apoptotic activity, the members of let-7 family, especially let-7c and let-7g suppressed the HCC. Expression of let-7c and let-7g was decreased in the hepatoma cells supplementary with BCL-xl protein [59]. In a current research, the significant p value of Let family members (Let-7c, Let-7g and Let-7i) in HCV patients with genotype 3a as compared to healthy individuals indicated their roles in the progression of HCV [2].

MiR-141

MiR-141 enhances the viral replication and hepatocellular carcimona in HCV infected patients by suppressing the expression of the DLC-1(a Rho GTPase-activating protein), a tumor suppressor gene. Fourfold increases in the MiR-141 had been observed in HCV transfected hepatocyte cultures with genotype 1a and 1b [6]. Anti-MiR-141 induction results in knockdown of MiR-141 showed inhibition of HCV replication thus had shown the dependence of HCV replication in MiR-141. Artificial induction of MiR-141 in HCV infected hepatocytes induced HCV replication, confirming its strong relation with the induction of HCV replication [6].

MiR-130

MiR-130 is the first reported mi-RNA that invades the interferon signaling pathway to increase the HCV replication [39]. Increase in the expression of MiR-130 is observed in liver biopsy of HCV infected hepatocytes. MiR-130 involves in modulation of HCV replication by targeting the IFITM (Interferon inducible trans-membrane protein). Knockdown by anti-MiR-130 has shown the reduction in the HCV replication [12]. Another study indicates the role of MiR-130a in the modulation of HCV replication by observing the overexpression of MiR-130 in liver biopsy of infected patients [82]. MiR-130 expression knockdown results in the overexpression of IFITM [12]. In contrary, an alternative study indicates the overexpression of MiR-130 helps in the inhibition of HCV replication by inducing innate immune system and activating interferon alpha and interferon beta [38]. Therefore the role of MiR-130 in the HCV modulation is still ambiguous. In a recent study on HCV induce HCC Egyptian patients reveal that miR-130 could not be used as a biomarker for early HCC. MiR-130 expression unregulated in both HCV induces HCC and HCV-associated chronic liver disease (CLD) [47].

Concluding remarks

This review demonstrates the important role of MiRNAs in Hepatitis C and hepatocellular carcinoma (HCC). MiRNAs have a significant role in the modulation of HCV. The up-

regulation and down-regulation of different MiRNAs in HCV samples indicates the role of these MiRNAs in promoting and inhibiting the HCV propagation respectively. Role of mi-RNAs in HCV is still ambiguous and requires further research to explain their function. These microRNAs can be used as biomarkers for the early detection of HCV propagation. Since MiR-122 has a main role in the propagation of HCV. So anti-MiR122 drug (MiRavirsen) has shown a reduction in the level of HCV and is in clinical phase II. In future, extensive study on MiRNAs related to HCV by microarray will be required that can help in predicting the HCV propagation and therapeutic treatments.

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