Competitive Binding between miR-122 and p68 onto Hepatitis C Viral RNA

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Background:
Liver-specific microRNA (miR)-122 has been shown to be involved in regulating translation of hepatitis C viral (HCV) RNA. This study aimed to explore the molecular mechanism of miR-122 in regulating HCV RNA translation initiation.

Material/Methods:
In human liver hepatocellular carcinoma cell line HepG2, UV cross-link assay was performed on a large scale to identify RNA-binding proteins with gradient concentrations of miR-122. Analytical ultracentrifugation was then used to separate the translation initiation complexes. All RNA-binding proteins were then identified by Western blotting.

Results:
The binding of 68 kDa protein (p68) to HCV RNA was suppressed by the addition of miR-122 via the competitive binding assay. Such inhibition can be eliminated by the addition of 2 ’-O-methylated oligonucleotides. This binding suppression was determined to be specific for miR-122, which used the mature single-stranded RNA to suppress the binding of p68 onto HCV RNA. This binding inhibition was further validated by using authentic miR-122 with conserved regions and mutated sequences.

Conclusions:
The binding of p68 onto HCV RNA can be specifically inhibited by miR-122 via a competitive binding process.

MeSH Keywords:
Hepacivirus • Proto-Oncogene Proteins c-rel • Vitamin D-Binding Protein

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Background

As an important step of eukaryotic gene expression, the translation of mRNA into proteins involves 3 stages: initiation, elongation, and termination. The initiation of translation starts with a complex process that leads to the binding of 80S ribosome onto the mRNA start codon, making it the rate-limiting step in the whole process of protein biosynthesis. Essentially all eukaryotic mRNAs are monocistronic single-stranded RNA molecules with a 7-methylguanosine (m7G) cap at their 5'-ends via a 5'-5'-triphosphate bridge to the first nucleotide. This cap plays a critical role in recruiting initiation factors to help the binding of small ribosomal 40S subunit onto mRNA. This m7G-cap also stabilizes mRNA molecules by suppressing nucleotide degradation and regulates mRNA entry to the cytoplasm from the nucleus. The m7G-cap site in mRNA is followed by a non-coding region with 50 to 100 nucleotide length, which is named the 5'-untranslated region (5'-UTR). The open reading frame (ORF) is downstream of 5'-UTR, flanked by the 3'-untranslated region (3'-UTR) and a poly-adenine monotone sequence with 100–250 bp length. This poly (A)-tail plays an important role in regulating translation and stabilizing mRNA. As an alternative to the m7G-cap, the translation can also be initiated from an internal ribosome entry site (IRES) at 5' end of mRNA, supporting the recruitment of ribosomal complexes at the start codon or its immediate vicinity.

The m7G cap-dependent translation initiation is a complex process involving several eukaryotic initiation factors (eIFs). The initiation complex, however, must be dissociated before the start of ribosomal translation via the binding of eIF1A and eIF3 onto the 40S ribosome subunit, thereby blocking the further accumulation of 60S subunits. A ternary complex consisting of methionine-loaded initiator tRNA (Met-tRNA) coupled to GTP, eIF2 and 40S ribosomal subunit forms the 43S pre-initiation complex [3]. The recruiting of this complex to the 5'-terminal m7G cap of mRNA is mediated by another complex consisting of eIF4E, eIF4G, and eIF4A, which are collectively called eIF4F. Within this complex, the adapter protein eIF4G forms the linkage between the pre-initiation complex and m7G cap via its binding to 40S subunit-bound initiation factor eIF3 [4]. The interaction of eIF4G with the mRNA poly (A)-binding protein (PABP) then circulates the whole mRNA molecule by bringing its 3'-UTR into close proximity with its 5'-UTR [5]. It is believed that the recycling of ribosomes and their recruitment to the 5'-UTR promotes and frees an intact 3'-end of mRNA [6].

Searching for the pre-initiation complex binding sites downstream of AUG triplet in mRNA requires the resolution of secondary structures of ATP-coupled RNA helicase eIF4A [7]. The initiation factors eIF1 and eIF1A are essential in this process for the precise binding onto the correct start codon [8]. If eIF2-GDP successfully helps the binding of mRNA onto Met-tRNA via the complementary base pairing, the eIF5-mediated GTP hydrolysis causes further dissociation of initiation factors. The translation initiation is then catalyzed by the recruitment of 60S subunit onto 40S subunit and dissociation of other initiation factors by the help of eIF5B [9]. Based on this information, the present study aimed to explore the molecular mechanism of miR-122 in regulating HCV RNA translation initiation.

Material and Methods

Cell culture

Human liver hepatocellular carcinoma cell line HepG2 was used in this study. The lack of endonuclease activity (ENDA) allows the preparation of high-quality plasmid DNA. The lack of recombination activity (recA) increases the stability of the insert. The mutated HSDR protects the cloned DNA from the EcoK endonuclease system. Recombinant plasmids were selected by the antibiotic resistance to tetracycline.

UV cross-link assay

In the UV cross-link assay, RNA-containing radiolabeled pyrimidine nucleotides were energetically excited by UV irradiation and reacted with amino acid side chains of proteins. Thus, the radioactive label is specifically transmitted to the covalent bonds of proteins, which then can be separated by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography. The amino acids exhibit crosslink reactivity following a decreasing order: from cysteine, tyrosine, phenylalanine, histidine, arginine, and cysteine, to serine and lysine. Subsequently, proteins were separated by 10% SDS-PAGE and stained. The gels were dried for 2 h and visualized by autoradiography.

Western blotting

To identify RNA-binding proteins using mass spectrometry, UV cross-link reactions were performed on a large scale. In order to accumulate sufficient amounts of target proteins, HepG2 cell extracts with the total protein amount at 1.2 to 1.8 mg were used. As the efficiency of cross-links between radiolabeled RNA and proteins is only about 5% to 10% [10], the corresponding amount of radioactively labeled RNA (200 pmol) was used in the crosslink reaction. The removal of unbound and RNase A-digested RNA was performed via subsequent 2D gel electrophoresis followed by purification using Trizol reagent (Invitrogen) according to the manufacturer’s instructions and separation by pHPLC using an Illustra NAPTM-5 column (GE Healthcare). The protein pellet was used in a further
2D gel electrophoresis or rpHPLC followed by mass spectrometric analysis.

**Analytical ultracentrifugation**

The component of translation initiation complexes was determined by analytical ultracentrifugation, as previously described [11]. The sucrose gradient buffer was prepared the day before centrifugation and was frozen at –20°C until use. Gradient buffers including 35%, 30%, 25%, 20%, 15%, and 10% sucrose (2 ml each in 50 mM Tris-HCl pH 8.4, 6 mM MgCl₂, 60 mM NaCl, and 10 mM DTT) were layered in centrifuge tubes. A uniform sucrose gradient buffer was prepared by repeated-ly freezing and thawing.

The cell lysate was applied onto the sucrose gradient solution, which was then centrifuged using an SW 40 rotor (Beckman, 5.5 h, 4°C, 200 000 g at the mean radius of the tube) and drained from the tube bottom in 0.5-ml fractions. In each fraction, 2 ml scintillator liquids (Roth) were added and the amount of emitted beta-radiation was recorded as counts per minute (cpm). Radioactive profiles were created by these radiation values, which were used to determine the amount of bound-ed RNA in complexes.

**Results**

**miR-122 attenuated the binding of p68 protein onto HCV RNA**

To investigate whether miR-122 alters the binding behavior of protein complex onto HCV RNA, we performed an RNA-protein binding assay using gradient concentrations of miR-122. Endogenously radiolabeled HCV RNA was prepared containing a short open reading frame (ORF), which was flanked by the 5'-UTR of HCV RNA with wild-type or mutated miR-122 target sequences plus the authentic 3'-UTR of HCV (Figure 1A). UV-crosslink assays showed that various proteins interact with both wild-type and mutant HCV RNA in HepG2 cells. The most prominent interaction existed between HCV RNA and a protein with the molecular weight around 68 kDa, as shown in SDS-gel migration bands (Figure 1B, 1C). As suggested by previous studies, this protein was identified as polypyrimidine

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Figure 3. The processing of miR-122 duplexes in cell extract is not sufficient to prevent the interaction of p68 with the HCV RNA. UV-crosslink experiments of radiolabeled HCV-RNA wild type (A) and the mutant HCV RNA (B).

tract-binding protein (PTB). No bands can be seen in the range of 97 kDa, perhaps due to binding of an Argonaute protein to the HCV RNA.

A second protein band was also identified as it localized in the molecular range of p68 kDa. The binding affinity of this protein to wild-type or mutant HCV RNA in the presence of large amounts of miR-122 was greatly reduced (Figure 1B, 1C). A 200-fold excess of miR-122 significantly decreased the intensity of p68 kDa protein band, and a 1000-fold miR-122 almost completely inhibited such protein binding onto either wild-type or mutant HCV RNA (Figure 1B, 1C). To test the binding specificity, cross-reaction was performed in the presence of mutated miR-122 or brain-specific miR-124. The binding of p68 kDa protein to HCV RNA could be suppressed as efficiently as those by the addition of miR-122 (Figure 1B, 1C). Therefore, the inhibition of protein binding onto HCV RNA is not caused by the addition of excess nonspecific competitors.

Furthermore, miR-122-induced sequence-specific inhibition on the binding between p68 kDa protein and HCV RNA can be prevented by anti-miR-122 strand with 2'-O-methylation (Figure 2A, 2B). This result provides further evidence that the aforementioned suppression of protein binding is miR-122-specific.

Another important finding was that only mature single-stranded miRNA reduced the binding of p68 kDa proteins onto HCV RNA. Such binding could not be suppressed by miR-122 duplex/mu-duplex or miR-124 duplex (Figure 3A, 3B). There are several possible explanations of these phenomena. Firstly, the incubation time of RNA and cell extracts may be too short to allow the formation of miRNA duplexes. Secondly, the protein extraction technique adapted from those in rabbit reticulocyte lysate [12] may not litigate the miRNA duplexes. This is supported by a previous study, which observed the conversion of exogenous pre-miR-let-7 into functional miRNA let-7 in cytoplasmic extracts of HEK293F cells by the overexpression of Ago1 or Ago2 [13], since Argonaute proteins function as key components of miRNP complexes along with other RNA-binding proteins, including the RNase III enzyme Dicer [14].

p68 interacts with 5’-UTR of HCV RNA

To investigate whether the inhibitory effect of miR-122 is caused by the direct complementary base pairing between miR-122 and 5’-UTR of HCV RNA and whether proteins at 5’-UTR of HCV RNA binds or interacts with its 3’-UTR region, further binding assays were performed using cytoplasmic cell extracts and HCV RNA with wild-type or mutated 5’-UTR (Figure 4C).

Autoradiography showed similar results with those observed in experiments using wild-type HCV RNA with intact 3’-UTR sequences (Figure 4A, 4B). It was strikingly shown that the dominant protein band of 57 kDa was no longer detectable by the deletion of 3’-UTR region of HCV RNA, suggesting the PTB nature of this protein. The p68 kDa protein band, however, was identified by the competition with miR-122, showing the interaction with 5’-UTR of HCV RNA.

The original hypothesis that miR-122 directly binds to its 2 target sequences in the HCV 5’-UTR to inhibit the RNA-protein binding is challenged by these results, because if the original model is correct, the binding of p68 kDa protein to mutated HCV RNA should be suppressed by mutated miR-122 but not by the authentic miR-122. Our results, however, showed that the inhibition of protein binding with the authentic miR-122 was observed under HCV RNA containing either conserved or mutated miR-122 target sequences (Figure 4A, 4B). In contrast, mutated miR-122 can suppress the binding of p68-kDa protein to wild-type or mutant HCV RNA only at 1000-fold excess concentrations. The results also suggest the competition between miR-122 and miR-122 mu or miR-124 in suppression of the interaction between p68 kDa protein and HCV RNA (Figure 4D).
Our results thus collectively suggest that the down-regulated binding of p68 kDa protein onto 5'-UTR of HCV RNA was likely caused by the competitive binding of proteins onto miR-122. Since the sequence of authentic and mutated miR-122 differ only in nucleotides 2 to 8, which is the so-called “seed” sequence, it may be such a sequence that determines the binding affinity onto p68 kDa protein, possibly via a slight preference of the protein onto 5'-UTR of wild-type miR-122.

**Discussions**

A possible mechanism of translation initiation by miRNA was studied as summarized above. The inhibited translation of HCV RNA by an RNA-RNA interaction between 5'-UTR and the coding region of HCV RNA in a chaperone-like conformation as those for miR-122 cannot be validated in the current stage. In contrast, such an inhibitory function of miR-122 has been confirmed in *in vitro* translation of HCV RNA in rabbit reticulocyte lysates.

In general, miRNAs form a complex with Argonaute protein and the “P-body” protein GW182 to suppress their target mRNAs. Interestingly, the number and size of “P-bodies” and the expression of GW182 in proliferating cells are increased compared to those in resting cells, largely explaining the loss of translational repression in quiescent cells [15]. As shown by Vasudevan and Steitz, cells under stress induced expression of tumor necrosis factor-α (TNF-α) by the recruitment of miRNP-associated factors Agonaute (Ago) 2 and fragile-X-mental-retardation-related protein 1 (FXR1) to AU-rich elements (ARE) of 3'-UTR of mRNA [16]. Therefore, the re-organization of miRNP complex by altering the protein composition exerts the positive effect of miRNA for regulating translation efficiency.

In the case of translational stimulation of HCV by miR-122, it is worth investigating which component interacts with miRNP complex onto 5'-UTR of HCV RNA and whether Ago proteins interact with other components. Since Ago 2 (eIF2C) was originally identified as an activator of translation initiation by stimulating the formation of ternary complexes, the modulation of miRNA function by Argonaute protein could not be ruled out, as it can intensify the formation of 48S initiation complexes onto HCV RNA.

In contrast to the m7G-cap-dependent initiation of translation of mRNA, binding of the small 40S ribosomal subunit to HCV
RNA IRES is independent of the cap-binding complex eIF4F, which consists of eukaryotic initiation factors eIF4G and eIF4A [19]. A study showed that the 40S subunit is bound by the HCV IRES, with high affinity a dissociation constant (K_d) = 2 nM [20]. This result leads to the question of how the 40S subunit is released from the IRES before the start of translation elongation. The miR-122 is a cellular factor that decreases the affinity of the HCV IRES to the 40S subunit by binding to target sequences upstream of the HCV IRES and inducing a conformational change. This would allow the accumulation of the 60S subunit followed by the transition into the 80S ribosome used in the elongation phase and accelerate the IRES element for further recruiting.

The formation and binding of a protein complex (“ON”), as well as its detachment from the binding site (“OFF”) and thus the transition into elongation, is crucial for efficient initiation and also underlies the mechanism of transcription initiation of RNA polymerase II [21]. The efficiency of transcription initiation is dependent not only on the formation of an initiation complex consisting of polymerase and transcription initiation factors that recognizes sequence on the DNA, but also the structural transformation of this complex, thereby allowing the exit of the promoter and the transition into elongation phase. This “escape promoter” process is the rate-limiting step in transcription initiation.

In the present study we show that the p68 protein binding to HCV 5'-UTR is inhibited specifically by miR-122. The decreased binding of this protein to the HCV IRES is not due to the displacement of the protein by interacting with its 2 target sequences in miR-122, as wild-type miR-122's binding affinity to HCV RNA with mutations in miR-122 target sequences was significantly decreased, whereas only a weak inhibitory effect could be achieved using mutated miR-122. Therefore, the protein might directly bind to miRNA sequence with a slight preference for wild-type miR-122.

The p68-kDa protein could be identified as a heat shock cognate 71 kDa protein. In this study, however, a validation via other methods and further characterization of the protein function associated with translation stimulation of HCV RNA by miR-122 was infeasible due to time constraints. The binding of Hsc70 to miR-122 has a functional significance and its potential relationship with miR-122-mediated stimulation of HCV translation should be examined for a reliable characterization of this protein.

The interaction of chaperone Hsc70 with the domain III of HCV IRES in cytoplasmic extract of non-hepatic cell lines HepG2 or BHK-21 has been previously reported [21]. The domain III mediates the interaction between 43S initiation complex and HCV IRES by direct binding of 40S ribosomal subunit and eIF3. The binding of Hsc70 to translation initiation site at HCV IRES domain is necessary. It is therefore conceivable that Hsc70 works through the interaction with miR-122 in HCV IRES to accelerate the formation of 48S initiation complexes. The functional relevance of Hsc70 in the stimulation of HCV translation receives further support from studies in which the recruitment of cellular chaperone network components for efficient viral replication has been demonstrated [23]. For example, an Hsp70 chaperone complex for the genome replication of human papilloma virus is indispensable because the chaperone complex is formed by re-modeling of protein components of the replication complex, thereby achieving efficient initiation of viral genome replication.

Conclusions

This study searched for factors that mediate the stimulatory effect of miR-122 on HCV translation and found a protein of about p68 kDa, whose binding to the HCV RNA was specifically suppressed by miR-122. This effect is not due to the displacement of the protein onto HCV RNA by binding of miR-122 to its target sequences in 5'-UTR of HCV RNA as the sequence-specific inhibition of miR-122 with the 2'-O-methylated strand suppressed by miR-122. This effect is not due to the displacement of the protein onto HCV RNA by binding of miR-122 to its target sequences in 5'-UTR of HCV RNA as the sequence-specific inhibition of miR-122 with the 2'-O-methylated strand or the prevention by anti-miR-122 oligonucleotide. The binding of p68 protein could be further demonstrated using the HCV-IRES unrelated FMDV-IRES.

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