Modulation of miR-122 on persistently Borna disease virus infected human oligodendroglial cells

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A B S T R A C T

Using RNAhybrid software we found the predicted binding of complementary sequences between miR-122 and viral mRNAs, may be important for the antiviral effect of miR-122 on Borna disease virus (BDV). A moderate expression of miR-122 was identified in human oligodendroglial cells (OL), but with a much lower level of miR-122 in BDV persistent infection (OL/BDV) and cells transfected with BDV gene expression vectors. The over-expression of miR-122 and specific blocking experiments demonstrated that miR-122 was able to specifically inhibit BDV protein synthesis, viral gene replication and transcription, and induce the secretion/synthesis of interferon (IFN) in OL and OL/BDV cells. The abolishment of miR-122 by AMO-122 inhibited endogenous IFN induction by IFN-beta. These results indicate that miR-122 can exert direct antiviral function by inhibiting BDV translation and replication on one hand, while acting indirectly through IFN to increase the host innate immunity to modulate the virus–host interactions on the other hand.

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1. Introduction

MicroRNAs (miRNAs) are a class of non-coding RNAs with 19–24 nucleotides, which play important roles in the cleavage and regulating the translation of target genes depending on the extent of matching of complementary sequences with the target mRNAs, and implicate in a number of biological processes including the host–virus interactions (Ambros, 2004; Bartel, 2004; Yeung et al., 2007).

Recent studies found that some miRNAs can inhibit viral replication by targeting the viral genome or mRNA, and suggest that miRNAs are integral components of host–virus interactions and such interactions are implicated in the processes and manners of viral infection and host response (Ghosh et al., 2009; Gottwein and Cullen, 2008). For example, host miRNAs could regulate negatively the viral replication and synthesis of murine cytomegalovirus (MCMV), primate foamy retrovirus (PFV), vesicular stomatitis virus (VSV) and HIV (Ahuwalia et al., 2008; Buck et al., 2010; Lecellier et al., 2005; Otsuka et al., 2007). However, the abundantly expressed liver-specific miR-122, promotes replication of HCV in cultured human hepatoma HuH7 cells stably expressing a HCV replicon (Jopling et al., 2005). To date, the question of how miR-122 modulates the antiviral effect in virus infection and host innate immunity remains largely unknown.

To investigate the antiviral effects of miR-122, we examined the most likely binding domains of miR-122 in a wide range of viruses using RNAhybrid software (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), and found that miR-122 can bind with complementary domains of a number of miRNAs of Borna disease virus (BDV) (Fig. 1). BDV, a nonsegmented negative-strand RNA virus, is considered as the causative agent of a number of neuropsychiatric diseases affecting animals and human (Cubitt et al., 1994; Lipkin et al., 2001). Prominent features of BDV infection include a strong neurotropism and the ability to establish noncytolytic persistent infection in animals as well as in cell lines of nervous system origin, which might be due to the influence of certain BDV proteins on the synthesis of IFN in cells (Unterstab et al., 2005). Therefore, we chose human oligodendroglial cell (OL) with BDV persistent infection (OL/BDV) or BDV genes transfected OL cells as model to investigate the role of miR-122 in modulating BDV persistent infection, and the interactions between virus infection and host innate immunity in the current study.

2. Materials and methods

2.1. Virus and cells

The BDV strain H1766, human oligodendroglial cells (OL) and BDV persistently infected OL cells (OL/BDV) used in this study
Fig. 1. Predicted binding sites of miR-122 with BDV P, N mRNAs. (A) Sequence of miR-122 with the seed sequence underlined. (B) The genome of BDV and its six major open reading frames (ORFs) in BDV genome sequence corresponding to the six proteins: Nucleoprotein (N), Phosphoprotein (P), Matrixprotein (M), Glycoprotein (G), RNA polymerase (L) and special protein (X). mRNA of BDV N and P genes with predicted miR-122 binding sites indicated in the 5’ end of BDV N mRNA and 3’ end of BDV P mRNA both on 2 positions.

were all obtained from Professor Kazuyoshi Ikuta of Japan. OL and OL/BDV cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% or 2% fetal bovine serum (FBS) respectively and 100 U/mL penicillin/streptomycin, in incubator at 37 °C in a humidified atmosphere of 5% CO2. Meanwhile, Huh7 cells and HepG2 cells, together with C6 glioma cells were cultured under routine conditions and used for comparison to evaluate the miR-122 level in OL cells. In addition, the expression vectors bearing BDV P and N gene sequences were generated by PCR amplification and cloned into the multiple cloning sites in the peGFP-N1 expression reporter vector. The sequences of all genes were further verified.

2.2. Synthesis of miRNA and miRNA inhibitor

The recombinant plasmid pDC-316-EGFP-U6-miR-122 was synthesized by Genscript, USA. The sequence of miR-122 inhibitor (anti-miRNA oligonucleotide, AMO-122) used in this study was the exact antisense copy of the mature miRNA sequence: 5’-CAGACACCAGUGCCCAUCCUCUCA-3’ for human miR-122. All the nucleotides in the AMO-122 containing 2’-O-methyl modification at every base were synthesized by Integrated DNA Technologies, USA. In contrast, miR-ctrl (miR-1) and AMO-ctrl (AMO-let7d*) were prepared and used as controls for evaluation of specific effects of miR-122 and AMO-122.

2.3. Transfection of miRNA plasmid, miRNA inhibitor and BDV gene expression vectors into OL and OL/BDV cells

After 24 h starvation in serum-free medium, OL and OL/BDV cells at 90% confluency were transfected with miR-122 plasmid, AMO-122 and the controls or BDV gene expression vectors with Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer’s instructions. Cells were harvested 48 h post-transfection for extraction of total RNA and proteins.

2.4. Quantification of BDV, miRNA and IFN

For the quantification of BDV genes and mRNA, conventional real-time RT-PCR was carried out using total RNA samples extracted from OL/BDV cells 48 h after transfection. Total RNA was isolated using Trizol (Invitrogen, USA) according to the manufacturer’s instructions. Briefly, total RNA was incubated with specific reverse primers (for viral genes) or oligodT (for viral mRNAs) and reverse transcriptase for 1 h at 42 °C. The enzyme was inactivated for 5 min at 95 °C. The cDNA was stored at −20 °C. Real-time PCR amplification was carried out to detect viral genes and mRNAs respectively. The cDNA of IFN and miR-122 were synthesized from total RNA using oligoDT and stem-loop reverse transcription primer, and then quantified by real-time PCR. Fold variations between RNA samples were calculated after normalization to GAPDH or U6. The sequences of the primers and probes (Hu et al., 2004; Schindler et al., 2007) used in current study are shown in Table 1.

2.5. Western blot analysis

Protein samples from OL and OL/BDV cells were extracted by cell lysis buffer (Beyotime, China), separated by SDS-polyacrylamide gel electrophoresis (12% polyacrylamide gels) and transferred to nitrocellulose membrane. The primary antibodies to BDV P or N protein and GAPDH were used for detection. The intensities of reaction products were recorded with Western blot Chemiluminescence Reagent Plus and expressed as a ratio to GAPDH.

2.6. Data analysis

Group data were expressed as mean ± s.e.m. Statistical analyses were carried out using SigmaStat (3.0). A p-value of <0.05 was taken as statistically significant.
3. Results

3.1. Prediction of miR-122 binding sequences in the BDV mRNAs

Based on the open reading frames of BDV genome, we examined the complementary sequence matchings of miR-122 (Fig. 1A) with BDV mRNAs of nucleoprotein (N), phosphoprotein (P), matrixprotein (M), glycoprotein (G), RNA polymerase (L) and nonglycobased special protein (X) (Fig. 1B), using RNAhybrid software with the minimum free energy (MFE) cutoff set at −22 kcal/mol. The results showed that miR-122 could bind specifically to the 5' and 3' end of target mRNAs of BDV encoding for N and P proteins, both on two positions (Fig. 1B). At the same time, the incomplete bindings of miR-122 to BDV mRNAs were also indicated. These results implicated that miR-122 could exert its antiviral effects by binding to BDV mRNAs.

3.2. Expression of miR-122 in OL cells with BDV persistent infection and in cells transfected with BDV gene expression vectors

In order to ascertain the level of miR-122 in OL cells, we first compared its expression level with hepatoma Huh7 and HepG2, glioma cells of C6 by real-time PCR using U6 as an internal control. The results showed that miR-122 was highly expressed in Huh7 but at a much lower level in HepG2, while the levels in OL and C6 cells were more moderately expressed but both higher than HepG2 cells (Fig. 2A). These results indicate that miR-122 is expressed at a detectable level in the glioma cell lines, and may play a role in cellular activities. We next examined the effects of BDV, BDV P and BDV N genes on the expression of miR-122. The results showed that miR-122 was expressed in both OL and OL/BDV cells; but the expression of miR-122 in OL/BDV cells was significantly lower than that in OL cells (p < 0.05) (Fig. 2B). The expression of miR-122 in OL cells transfected with BDV N and P gene expression vectors were significantly lower than the empty vector control (Fig. 2B). These results indicated that BDV persistent infection and BDV P and N proteins were able to suppress the expression of miR-122. We further transfected miR-122 plasmid and AMO-122 to OL and OL/BDV cells respectively to investigate the effects of miR-122. The results showed that the levels of miR-122 in transfected OL and OL/BDV cells were significantly higher than the plasmid control, and the up-regulated expression of miR-122 could be abolished by treatment of AMO-122 (Fig. 2C). The effects were most prominent after 48 h and lower, though still at high level by 72 h post-transfection. Thus 48 h post-transfection was used in all subsequent experiments.

3.3. Inhibitory effect of miR-122 on BDV translation and replication in OL cells with BDV gene transfection and in OL/BDV cells

Due to the fact that miRNAs are playing dominant roles on the repression of mRNA translation and cleavage of target genes and mRNAs, the effects of miR-122 on BDV translation and replication were examined in this experiment. BDV N or P gene transfected OL cells were firstly used to check the inhibitory effects of miR-122 on BDV translation by Western blot. The results showed that BDV P proteins were able to suppress the expression of miR-122. We further transfected miR-122 plasmid and AMO-122 to OL and OL/BDV cells respectively to investigate the effects of miR-122. The results showed that the levels of miR-122 in transfected OL and OL/BDV cells were significantly higher than the plasmid control, and the up-regulated expression of miR-122 could be abolished by treatment of AMO-122 (Fig. 2C). The effects were most prominent after 48 h and lower, though still at high level by 72 h post-transfection. Thus 48 h post-transfection was used in all subsequent experiments.
Fig. 2. Expression of miR-122 in OL and OL/BDV cells and repressed by BDV persistent infection, certain proteins of BDV and AMO-122. (A) A moderate level of miR-122 was detected in OL cells, which was lower than that of Huh7, but higher than HepG2 cells. (B) miR-122 expressed in OL and OL/BDV cells. Note the dramatic drop of miR-122 in OL/BDV compared to OL cells. After transfection of BDV N and P gene expression vectors to OL cells, the levels of miR-122 were significantly decreased compared with OL cells transfected with empty control vector. (C) In OL and OL/BDV cells, miR-122 was higher expressed by transfection of miR-122 plasmid and abolished specifically by treatment of AMO-122. Note that the effects were most significant at 48 h time point. *p < 0.05.

P protein but not N protein were further demonstrated in OL/BDV cells treated by miR-122, which was specifically inhibited by AMO-122 (Fig. 3C and D). However, the expression of the BDV P protein was not changed in OL/BDV cells after the treatment of miR-ctrl and AMO-ctrl (Fig. 3C and D), suggesting that miR-122 specifically inhibited the BDV P protein translation. This suggests that miR-122 may affect BDV P protein synthesis, which might be due to the post-transcriptional regulation of miRNA with complementary matching between miR-122 and BDV P mRNA.

On the other hand, to determine whether changes in miR-122 expression have any effect on BDV replication and viral RNA stability, real-time PCR was used to detect the changes of nucleic acids of BDV after transfection of miR-122 plasmid and AMO-122. The results showed that the level of BDV N gene and its mRNA were significantly repressed after the expression of miR-122 plasmid, and the depressed levels were reversed after treatment by AMO-122 (Fig. 3F). In the meantime, we found that the transfected miR-122 plasmid had limited effect on BDV P gene and its corresponding mRNA in OL/BDV cells (Fig. 3E). Moreover, the full or slight recovery of expressions of BDV N gene and mRNA were observed after treatment of AMO-122 (Fig. 3E and F). The specificity of miR-122 and AMO-122 affecting the BDV transcription and replication were confirmed with the treatment of miR-ctrl and AMO-ctrl, the latter had little influence on miR-122 (Fig. 3E and F). These further indicate that miR-122 may also affect viral replication and transcription in addition to post-transcription regulation to exert its specific anti-BDV effect.

3.4. The effect of miR-122 on the induction of IFN in OL and OL/BDV cells

To examine further that in addition to its direct antiviral effect, whether miR-122 plays any role in innate immunity of cells against viral infection, we first examined the expression of IFN type I in OL and OL/BDV cells, followed by transfection of miR-122 plasmid to these cells. The results showed that the expression levels of IFN-alpha and IFN-beta in OL/BDV cells were much lower than those of OL cells (Fig. 4A and B). The low level of IFN type I in OL/BDV cells may indicate a reduced host defense capability and the ability to remove the virus from the cells, thus, may be involved in the establishment of persistent infection of the affected cells.

However, when OL and OL/BDV cells were transfected with miR-122 plasmid, the expression levels of IFN type I were greatly enhanced compared to the cells transfected with miR-ctrl plasmid.
Fig. 3. Inhibition of miR-122 on the BDV translation, replication and transcription in OL/BDV cells. (A) There was significant inhibitory effect of miR-122 on BDV P protein synthesis in OL cells after cotransfected the miR-122 plasmid and BDV P gene expression vector. (B) There was no significant inhibitory effect of miR-122 on BDV N protein synthesis in OL cells after cotransfection of miR-122 plasmid and BDV N gene expression vector. (C) miR-122 had a significant inhibition on BDV P protein in OL/BDV cells after transfection of the miR-122 plasmid. (D) BDV N protein level was not obviously influenced by miR-122 in OL/BDV cells after transfected by miR-122 plasmid. (E) BDV P gene and mRNA were not significantly repressed in OL/BDV cells after transfected by the miR-122 plasmid. (F) BDV N gene and mRNA were significantly repressed after the expression of miR-122 plasmid in OL/BDV cells. *p < 0.05.
Fig. 4. The specific induction of IFN type I in OL and OL/BDV cells by miR-122 transfection. (A) The expression of IFN-alpha was significantly increased in OL and OL/BDV cells after the transfection of miR-122 plasmid. miR-ctrl had no effects on IFN-alpha expression. (B) The expression of IFN-beta was significantly increased in OL and OL/BDV cells after the transfection of miR-122 plasmid. Note that miR-ctrl had no effects on IFN-beta expression. *p < 0.05.

(Fig. 4A and B), suggesting that miR-122 may be an important factor to regulate specifically the secretion/synthesis of IFN in OL/BDV cells.

3.5. The inhibition of AMO-122 on the expression of endogenous IFN in OL and OL/BDV cells stimulated by IFN-beta

Meanwhile, to investigate the involvement of miR-122 in the IFN induction, we found the significant increased IFN type I expressions in OL and OL/BDV cells after the transfection of miR-122 (Fig. 4A and B). Whereas the induction ability of IFN-beta on IFN type I (especially IFN-beta) secretion/synthesis was fully abolished by AMO-122 treatment, but not in AMO-ctrl (Fig. 5A and B). All these results indicate that miR-122 may be a necessary intracellular regulator for the endogenous IFN secretion/synthesis stimulated by IFN treatment, thus, may be involved indirectly in IFN therapy for virus infection to exert its anti-BDV effects.

4. Discussion

To date the studies on antiviral effects of host miRNAs have mainly been focused on the complementary binding of miRNAs to the coding or non-coding sequences of the viral gene and mRNA, resulting in the inhibition of viral translation, transcription and replication (Bartel, 2004; Lecellier et al., 2005; Scaria et al., 2006). For example, miR-507 and miR-136 are targeting on Polymerase B2 (PB2) and Hemagglutinin (HA) genes of influenza virus to inhibit influenza virus replication (Scaria et al., 2006). Over-expression of host’s miR-24 and miR-93 suppressed VSV multiplication by approximately 50% in comparison with the control, through bind-
The P protein is a central regulatory element of BDV replication. The active polymerase complex of BDV is composed of L, P and N. Plasmid. Like in other members of the Mononegavirales family, with cotransfection of BDV P gene expression vector and miR-122, results from our current study indicated that miR-122 could exert the mechanism of miR-122 against BDV infection. Taken together, we believe that endogenous miR-122 can influence IFN secretion/synthesis.

Most miRNAs are expressed ubiquitously in various tissues, whereas a few appear to be more highly expressed in a tissue-specific manner (Lagos-Quintana et al., 2002; Sempere et al., 2004). Evidence to date suggests that miR-122 is specifically expressed in high levels in liver and cells derived from liver despite its modest expression in other tissues; it has thus been regarded as liver-specific miRNA (Chang et al., 2004; Lagos-Quintana et al., 2002). In HCV infected liver cells, it has been reported that there is an association between low pre-treatment miR-122 levels and a poor response to IFN therapy (Sarasin-Filipowicz et al., 2009). However, some reports have shown that miR-122 is essential for the replication of HCV and inhibition of miR-122 has been proven to be effective in controlling the HCV infection (Lanford et al., 2010).

Although miR-122 was considered to be highly expressed in liver, the results of our study indicated that miR-122 was also expressed in OL and OL/BDV cells. Moreover, the expression of miR-122 in OL cells was found to be sandwiched between hepatoma Huh7 and HepG2 cell lines in current study, being lower than Huh7 and higher than HepG2. Meanwhile, miR-122 expression in OL/BDV cells as detected by microarray analysis was several fold higher than a brain specific miRNA (i.e. miR-124) (Silber et al., 2008), which is believed to be playing a regulatory function in the brain or glioma cells (data not shown). Although a lower level of miR-122 was found in OL cells with BDV persistent infection and BDV gene transfection, it did not hamper the effectiveness of miR-122 in antivirus and regulation of innate immunity of OL cells in current study. Consequently, the innate immunity of host cells with respect to modulation of secretion/synthesis of IFN may also be different after virus infections, suggesting that miR-122 may have a much wider implication than what is currently known.

5. Conclusions

In summary, our results demonstrated that miR-122 might serve as an important endogenous gene regulatory factor which can act directly to inhibit virus replication and translation on one hand while indirectly to activate the host innate immunity to exert its antiviral effect on the other hand. Our result of BDV's ability to inhibit miR-122 expression further suggests that miR-122 may play an important role in the interaction between virus and host cells, which may be crucial in the establishment and removal of persistent infection of BDV in OL cells and affecting the effectiveness of IFN therapy for virus infection by altering the viral replication and host innate immunity in these cells.

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