Brief Report

Construction and Identification of a Human Liver Specific MicroRNA Eukaryotic Expression Vector

Shanshan Chen[1], Ming Ni[2, 4], Bing Yu[1, 4], Tingting Lv[1], Mengji Lu[1] and Feili Gong[3]

MiR-122 is one of the non-coding RNAs which showed its effects on the lipo-metablism, virus infection and HCC forming through regulation of liver gene expression. Its eukaryotic expression vector was constructed by using pSuper which was widely applied in the siRNA expression. The precursor of human miR-122 gene was amplified by polymerase chain reaction (PCR) from the human genomic DNA. The positive clones were screened by PCR and restriction enzyme digestion. The new expression vector of miR-122 was named pHsa-m122. PHsa-m122 and its controls were transfected to HepG2 cells. The miR-122 expression activity was evaluated by GFP122i sensor reporter plasmid through fluorescence detection and Western blot. It was shown that the fluorescence intensity of GFP122si and pHsa-m122 co-transfection group was weaker than that of the controls, so the functional activity of expressed miR-122 was detected. When HepG2 cells were co-transfected with HBV1.3 and pHsa-m122 plasmids, the results showed miR-122 may down-regulate the gene expression of HBV. The human liver specific microRNA eukaryotic expression vector of miR-122 was constructed successfully, which may facilitate further study of its function in the development of liver virus infection diseases and HCC. *Cellular & Molecular Immunology*. 2007; 4(6):473-477.

Key Words: miRNA, miR-122, eukaryotic expression vector, pSuper

Introduction

Mature microRNAs are short noncoding single-stranded RNAs that can posttranscriptionally regulate gene expression in plants and animals (1). They are about 22 nt non-coding small RNAs and important players in cellular proliferation and development. The mature miRNAs use a mode of silencing similar to that employed by siRNAs which cleave mRNA transcription (2-4). Although it is known that the human genome contains hundreds of microRNA genes and each miRNA can regulate a large number of mRNA targets, the overall effect of miRNAs on mRNA tissue profiles has not been systematically elucidated (5). Some investigators found microRNA may play a critical role in the development of virus infection diseases and tumorigenesis (6-8). MiR-122 is one kind of miRNA which specially expressed 70% of the total miRNA in mature human liver. MiR-122 located on human 18th chromosome, marked 18q21.31 (9). Since pSuper has been used in the siRNA and microRNA expression vector construction (10), miR-122 may be well expressed by using this vector. In the present study, we wanted to construct a eukaryotic expression vector of miR-122 and establish the basis for the future research.

Materials and Methods

Plasmid and reagents

The pSuper plasmid was preserved by the Division of Clinical Immunology of Tongji Hospital; the GFP122 sensor and GFP124 sensor were presented as a gift by Prof. Sarnow from the Department of Microbiology and Immunology of Standford University. *E. coli* DH5α and HepG2 cells were preserved in our lab; Taq enzyme and T4 ligase, restriction enzyme, marker, plasmid exacting kit was purchased from Takara and HBI Company. Anti-GFP monoclonal antibody and Lipofectamine 2000 were purchased from Sigma and Invitrogen, respectively. The HBsAg and HBeAg diagnostic ELISA kits were purchased from Shanghai Kehua Bio-

[1]Department of Pathogenic Biology, Tongji Medical College of Huazhong University of Science & Technology, Wuhan 430030, China;

[2]Department of Infectious Diseases, Tongji Hospital, Tongji Medical College of Huazhong University of Science & Technology, Wuhan 430030, China;

[3]Department of Immunology, Tongji Medical College of Huazhong University of Science & Technology, Wuhan 430030, China;

[4]Corresponding to: Dr. Bing Yu, Department of Pathogenic Biology, or Dr. Ming Ni, Department of Infectious Diseases, Tongji Hospital; Tongji Medical College of Huazhong University of Science & Technology, 13 Hangkong Rd, Wuhan 430030, China. Tel: +86-27-8365-0550, E-mail: andybing666@hotmail.com or niming@tjh.tjmu.edu.cn

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engineering Co., Ltd.

Bacteria and cell line

E. coli DH5α was grown in LB medium containing 50 mg/L ampicillin. Human HepG2 cell line was grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) at 37ºC, in 5% CO_2.

PCR amplication of pre-miR-122 gene fragments

Genomic DNA of pre-miR-122 was extracted as previously described. According to the complete DNA sequence of pre-miR-122 published and multiple clone sites of pSuper, the primers to amplify pre-miR-122 containing *BamH* I site in P1 and *Sal* I sites in P2 were designed, respectively. (P1: 5'-GCG GAT CCA TCA GAT GAA CCT TCT TGC T-3'; P2: 5'-GCG TCG ACA AAA ATT TCT CTG CTT AGG TCA CAA-3'). The target sequence was amplified by PCR under 94°C for 4 min; 94°C for 45 s, 60°C for 45 s, 72°C for 45 s, 30 cycles.

Construction of recombinant pHsa-m122 and sequencing analysis

PCR fragments of *BamH* I and *Sal* I-digested were inserted into the *Bgl* II and *Sal* I-digested site of eukaryotic expression vector pSuper. However, these sites were right next to each other and it was impossible to get much cutting by the second enzyme once the first enzyme has cut. This resulted in an extremely high background (15). The recombinant pHsa-m122 was confirmed by restriction enzyme digestion by *EcoR* I and *Sal* I. The correct sample was sent to Shanghai Invitrogen Company for the sequence identification.

GFP fluorescence assay

The cells were transfected in the 12-well plates in serum-free DMEM by using Lipofectamine 2000 (Invitrogen). GFP122si was the reporter plasmid of miR-122, and it could express the mRNA of GFP containing the sequence related to miR-122 in its 3'UTR. miR-122 could suppress the expression of GFP by binding to mRNA's 3' UTR, so it could be the excellent tool to detect the expression of miR-122. GFP124si was the reporter plasmid of miR-124, which was used as a control here. Cells were harvested 24 h after transfection and assayed for GFP by fluorescence microscopy and FACS after 36-h transfection.

Western blot

For the Western analysis total protein extracts from HepG2 cells were used. Forty-eight hours after transfection, the cells were lysed with EBC buffer (50 mM Tris pH8, 170 mM NaCl, 0.5% NP 40, 50 mM NaF) supplemented with protease inhibitors. Protein lysate (30 μg) was separated by 10% SDS-PAGE and transferred to PROTRAN nitrocellulose membrane in 25 mM Tris, 200 mM glycine, 20% methanol, at 30 V overnight at 4°C. After blocking nonspecific binding sites with 5% nonfat milk in PBS/Tween, the membrane was incubated for 2 h at RT with anti-GFP antibody (monoclonal,

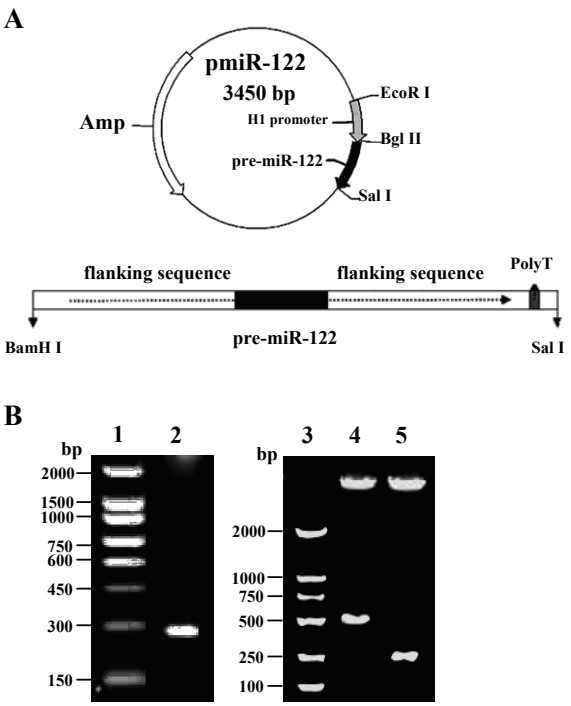

Figure 1. The construction of pHsa-m122 vector. (A) Schematic map of pHsa-m122 vector construction. (B) Left, the precursor of human miR-122 gene was amplified by PCR. Lane 1, DNA ladder (HBI DL2000 II). Lane 2, PCR product of the precursor miR-122 (270 bp). Right, the recombinant plasmid pmiR-122 was confirmed by double restriction endonuclease digestion. Lane 3, DNA ladder (DL2000); Lane 4, pHsa-miR-122 digestion with *EcoR* I and *Sal* I. The size of the small fragment was 520 bp; Lane 5, pSuper digestion with *EcoR* I and *Sal* I. The size of the small fragment was 250 bp.

diluted 1:300) and then for 1 h with the anti-rabit antibody (alkaline peroxidase-conjugate, diluted 1:2,000). After washed in PBS/Tween, the detection was performed.

ELISA measurements of HBsAg and HBeAg

Forty-eight hours after co-transfection of miR-122 and HBV 1.3 to HepG2 cells, the HBsAg and HBeAg in the culture media were measured by a diagnostic ELISA kit, according to the instructions.

Statistical analysis

In three repeated different experiments, the data obtained were statistically evaluated by analysis of variance (ANOVA) and comparison of means (Student's *t* test) at a significance level of $p < 0.05$. Both analyses were carried out using SPSS 13.0 statistical software.

Results

Recombinant plasmid was confirmed by restriction enzyme digestion

Compared with the chemical synthesis or transcription

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  1  CCTAAGCCTC CGCCTCCTCT TCCTCCATCC GCCCCGTCTC TCCCCCTTGA
 51  ACCTCCTCGT TCGACCCCGC CTCGATCCTC CCTTTATCCA GCCCTCACTC
101  CTTCTCTAGG CGCCGGAATT AGATCGATCT CGAGGTCGAC AAAAATTTCT
151  CTGCTTAGGT CACAATATGT GGAGCTGACA AGGTTCCCCT ATTATCAGTG
201  ACAATGGTGG AATGTGGAGG TGAAGTTAAC ACCTTCGTGG CTACAGAGTT
251  TCCTAGCAGA GCTGTGGAGT GTGACAATGG TGTTTGTGTC TAAACTATCA
301  AACGCCATTA TCACACTAAA TAGCTACTGC TAGGCAATCC TTCCCTCGAT
351  AAATGTCTTG GCATCGTTTG CTTTGAGCAA AAGGTTCATC TGATGGATCT
401  GTGGTCTCAT ACAGAACTTA TAAGATTCCC AAATCCAAAG ACATTTCACG
451  TTTATGGTGA TTTCCCAGAA CACATAGCGA CATGCAAATA TTGCAGGGCG
501  CCCTCCCTGT CCCTCACAGC CATCTTCCTG CCAGGGCGCA CGCGCGCTGG
551  GTGTTCCCGC CTAGTGACAC TGGGCCCGCG ATTCCTTGGA GCGGGTTGAT
601  GACGTCAGCG TTCGAATTCT ACCGGGTAGG GAGCGCTTTT CCCAAGGCAG
651  TCTGGAGCAT GCGCTTTAGC AGCCCCGCTG GGCACTTGGC GCTACACAA
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Figure 2. Sequences of the recombinant plasmid pHsa-m122.

mature miRNA *in vitro*, construction of the eukaryotic expression vector should be more economic, and applied in long-term research of its biological functions. Both mature siRNA and miRNA are precursor sequences cutting by RNase III enzyme DICER to form ~ 22 nt small molecule RNA. This process is very similar between siRNA and miRNA. So siRNA expression vectors were current extensively used in expression miRNA molecules. The construction process was shown by schematic map (Figure 1A). After pHsa-m122 was digested by *BamH* I and *Sal* I, a 270 bp fragment of pre-miR-122 was directly cloned into pSuper vector. In the successfully constructed vector pHsa-m122 the *Bgl* II and *Sal* I sites were impossible to get much cutting. So other restriction enzymes *EcoR* I and *Sal* I were selected to demonstrate that recombinant plasmids contained the pre-miR-122 genes. Restriction enzyme digestion products were analyzed on agarose gel (Figure 1B).

Sequence analysis of pHsa-m122

The positive clones were sent to Shanghai Yingjun sequencing Company, sequencing code CS070321060. The correct inserted fragment was shade in grey (Figure 2). In both sides of the miRNA sequence, there were correct enzyme sites *EcoR* I and *Sal* I. The results matched the schematic map of pHsa-m122 vector construction. After sequences BLAST in Genebank, it was confirmed that miR-122 precursor sequences were successfully cloned into the eukaryotic expression vector pSuper.

Cell transfection and GFP reporter gene expression assay

After transfection, HepG2 cells were harvested and assayed for GFP by fluorescence microscopy and FACS. Twenty-four hours after co-transfection of pHsa-m122, GFP122si or plasmid GFP124si in HepG2 cells, fluorescence microscopy analysis showed that in GFP122si and pHsa-m122 co-transfected group, fluorescence intensity of GFP was obviously weaker than that of controls. These result suggested that the correct expression of miR122 down-regulated the expression of GFP122si (Figure 3).

Thirty-six hours after transfection, HepG2 cells were collected to perform FACS assay. As indicated in Figure 3,

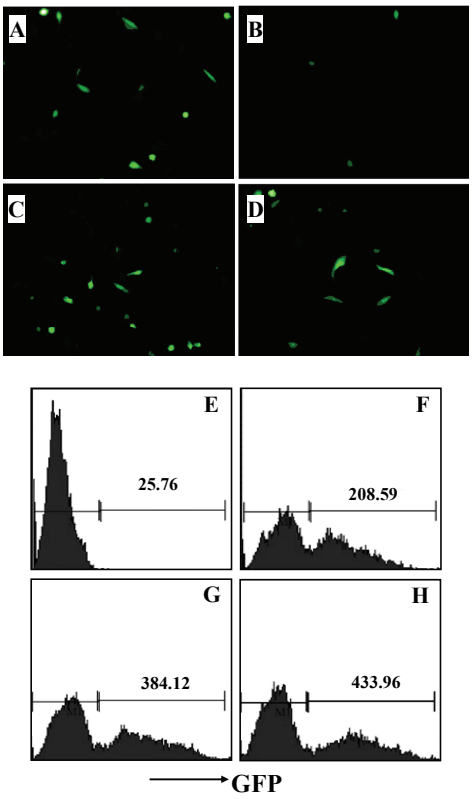

Figure 3. Down-regulation effects of the pHsa - m122 expression of miR-122. Expression of green fluorescent protein in HepG2 cells was detected by fluorescence microscopy (200×) (upper). (A) positive control; (B) co-transfection of GFP122si and pHsa-m122 plasmids; (C) negative control (co-transfection of GFP122si and pSuper plasmids); (D) no relation control (co-transfection of GFP124si and pHsa-m122 plasmids). GFP fluorescence was analyzed by FACS (lower). Results were shown as the mean fluorescence intensity (MFI), which represented the average level of GFP surface expression on the total successfully transfected cells. (E) blank control (no transfection); (F) co-transfection GFP122si and pHsa-m122 plasmids; (G) negative control (co-transfection GFP122si and pSuper plasmids); (H) no relation control (co-transfection GFP124si and pHsa-m122 plasmids).

the mean fluorescence intensity (MFI) of the GFP122si and pHsa-m122 co-transfected cells was obviously lower than those of other control groups. The result further confirmed that miR-122 expressed intrabody could significantly down-regulate the GFP122si expression.

Forty-eight hours after transfection, GFP protein expression was detected by Western Blot. As shown in Figure 4, GFP protein significant decrease was observed in pHsa-m122 transfected cells. This result indicated that the expression of miR-122 played a functional role.

ELISA measurements of HBsAg and HBeAg

To evaluate the effects of HBV miRNA on HBV gene expression, HBsAg and HBeAg concentrations in the culture media of treated and control cells were measured 48 h after transfection using a diagnostic ELISA kit. The concentrations

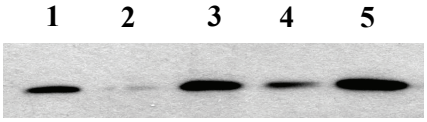

Figure 4. The expressions of GFP were detected by Western blot. Lane 1, transfection of GFP122si plasmid; Lane 2, co-transfection of GFP122si and pHsa-m122 plasmids, Lane 3, transfection of GFP124si plasmid; Lane 4, co-transfection of GFP124si and pHsa-m122 plasmids; Lane 5, co-transfection of GFP122si and pSuper plasmids.

of HBsAg and HBeAg were decreased by co-transfection of HBV1.3 and pHsa-m122 plasmids or HBV psiRNA plasmids treatment compared with negative controls (Figure 5).

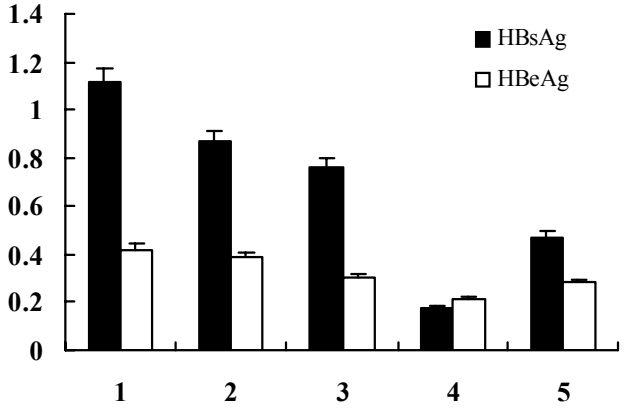

Figure 5. ELISA measurements of HBsAg and HBeAg. (1) Co-transfection of HBV1.3 and pSuper plasmid; (2) co-transfection of HBV1.3 and GFP122si plasmids; (3) co-transfection of HBV1.3 and GFP124si plasmids; (4) co-transfection of HBV1.3 and pHsa-m122 plasmids; (5) co-transfection of HBV1.3 and HBV psiRNA plasmids.

Discussion

More than 700 human miRNAs have been identified so far and more than 200 miRNAs are correlated with cancer or virus infection diseases (11). The liver miRNA expression profile is dominated by a single sequence, miR-122. With approximately 50,000 copies per cell, the liver serves as an ideal site for the study of miRNAs. MiR-122 was down-regulated in the majority of HCCs and in all examined HCC-derived cell lines. The high frequency of aberrant regulation of these miRNAs in HCC versus nontumor liver suggests that they might play an important role in hepatocarcinogenesis. In addition, microRNAs (miRNAs) represent a vital component of the innate antiviral immune response (12). Some investigators found that IFN-β down-regulates the expression of liver-specific miR-122. Therefore, it indicated miRNAs attack viral infections through the interferon system. The relationship between miR122 and liver immunity remained elusive. Lewis et al. predicted the probable target of miR-122 by the computer statistics and bioinformatics, including the CAT-1 (cationic amino acid transporter), hepatoviruses and other genes which can be down-regulated during the proliferation of hepatic cells (13, 14).

We explored the possibility of synthesizing miRNAs in mammalian cells by an expression vector. This method showed inexpensive and could be used in long-term experiments. The pSuper-based vectors developed in the present study provide an effective mechanism for introduction of the miRNAs into cultured cells. It provides a mammalian expression vector that directs intracellular synthesis of small RNA-like transcripts. The vector uses the polymerase-III H1-RNA gene promoter, as lacking a polyadenosine tail and has a well-defined start of transcription and a termination signal consisting of five thymidines in a row (T5).

It has been designed to express two 19 nucleotide reverse complement homologous oligomers to a portion of the target gene of interest separated by a short spacer region which has no homology to either of the 19 nt sequences. The hairpin RNA is a good substrate for the cellular enzyme Dicer which processes the hairpin to generate a short interfering RNA with 19 base pairs homologous to the target gene, each strand having a 3' overhang of two uridine residues. This structure has been suggested as being optimum for mediating RNAi in mammalian cells. The vector has successfully been used to validate the function of many genes. The functional activity of miR-122 expression was confirmed by GFP122 sensor reporter plasmid. GFP122si contains GFP gene and 3'UTR with miR-122 fully complementary sequence. MiR-122 may effectively inhibit GFP122si expression, so GFP protein expression decrease. GFP122si could be used as miR-122 expression testing tools. Green fluorescent protein expression does not have host cell toxicity, and has been widely used as reporter gene (15). Some experiments confirmed that the flanking sequences besides the mature miRNA should be necessary for miRNA expression (16). The miRNA expression vector has higher expression efficiency with the insertion sequence of miRNA precursors (17). That is because Drosha enzymes take part in the identification of some of pri-miRNA precursor sequence. If recognition sequence integrity, more efficient expression of the corresponding miRNAs may be expressed higher. In this study, the results showed efficiency expression of functional miR-122.

Our results also showed miR-122 may down-regulate the gene expression of HBV when co-transfected with HBV1.3 and pHsa-m122 plasmids. It was proved that miR-122 may regulate IFN-related gene expression to influence HBV replication, but the mechanism needs further study. The construction of eukaryotic expression vector of miR-122 may facilitate further study in the development of liver virus infection diseases and HCC. This simple construction and identification method may have broad application in other microRNAs research.

Acknowledgements

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