Effects of miR-9 and Tetramethylpyrazine on Activation of Hepatic Stellate Cells

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Micro-RNAs (miRNAs) are involved in regulation of the incidence and development of several hepatic diseases. Thus manipulating miRNAs may be a promising therapeutic strategy against these entities. In this study hepatic stellate cells (HSCs) were transfected with hsa-miR-9 or anti-hsa-miR-9, treated with tetramethylpyrazine (TMP), or subjected to treatment with TMP and hsa-miR-9 transfection (combined treatment group). Then, real-time polymerase chain reaction (PCR) was performed to measure mRNA levels of hsa-miR-9. Expression of hsa-miR-9 was highest in the combination treatment group compared with other groups, and significantly higher than TMP-treated and hsa-miR-9-transfected groups (both p<0.05). The anti-hsa-miR-9-transfected group expressed the lowest mRNA level of hsa-miR-9 with marked decrease versus control (p<0.05). Downstream factors that may be affected by miR-9 such as leptin, α-smooth muscle actin (SMA), and collagen I, as well as phosphorylation levels of Janus kinase 1 (JAK1)/signal transducer and activator of transcription 3 (STAT3) were investigated at the protein level. All these factors were regulated contrariwise to expression trends of hsa-miR-9, showing the lowest level in the combination treatment group and highest level in anti-hsa-miR-9-transfected group. These results suggest that both transfection of hsa-miR-9 and TMP can lead to upregulated endogenous expression of hsa-miR-9, inhibit activation of JAK1/STAT3 signal pathway induced by leptin, and lead to reduction of α-SMA and collagen I—thus impeding activation of HSC.

Key words hsa-miR-9; leptin; tetramethylpyrazine (TMP); hepatic stellate cell (HSC); Janus kinase 1 (JAK1)/signal transducer and activator of transcription 3 (STAT3)

MATERIALS AND METHODS

Cell Culture The human immortalized HSC, LX-2 cell line, was provided by Zhejiang University School of Medicine. LX-2 cells exhibited typical features of HSC in primary culture, such as expression of desmin, glial acidic fibrillary protein, and responsiveness to transforming growth factor-β1 (TGF-β1). LX-2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with 5% fetal bovine serum (FBS) (Gibco), 100U/mL penicillin (Sigma), and 100µg/mL streptomycin (Sigma) and incubated at 37°C in a humidified atmosphere with 5% CO2. Transfection of Cells with miRNA and Treatment with TMP 5×10^5 cells/well were seeded in 6-well plate, then transfection was performed using siPORT NeoFX reagent (Applied Biosystems) according to manufacturer’s instructions. Five groups and 6 repeats in each group were designed in this study, including control, synthetic hsa-miR-9-transfected, anti-hsa-miR-9-transfected, TMP-treated, and combined treatment group with synthetic hsa-miR-9-transfected and TMP (Wu xi, China). Twenty four hours after treatment, the

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cells were washed and new medium was added. Combined treatment group was transferred with hsa-miR-9 and treated with the final concentration of TMP (200 mg/L). Sequence for hsa-miR-9: 5'-GUA GCA CCA UCT GAA AUC GGU UA-3' (Sangon Biotech, Shanghai). The cells were collected after incubation for 36 h and the corresponding fibrotic phenotype were observed by the optical microscope. All the cells were collected and stored at −80°C until use.

miRNA Isolation and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) for miRNA Assay miRNA was isolated from LX-2 cells using the mirVana miRNA isolation kit (Ambion, TX, U.S.A.) and first-strand cDNA was synthesized using the NCode miRNA First-Strand cDNA synthesis kit (Invitrogen, CA, U.S.A.) in ABI Prizm 7500 machine. Sequence-specific primer was designed according to NCode miRNA-A First-Strand cDNA synthesis kit guidelines, hsa-miR-9 sense primer: 5'-GTA GCA CCA TCT GAA ATC GGT TA-3', 5'SrRNA antisense primer: 5'-GTC TAC GGC CAT ACC CTG AA-3'. Quantitative real-time PCR was performed according to manufacturer’s instructions and miRNA was normalized to 5S rRNA.

RNA Isolation and qRT-PCR for mRNA Assay Total RNAs were isolated from cells using total RNA isolation kit (Qiagen, U.S.A.), and cDNA was synthesized using the iScript cDNA synthesis kit according to manufacturer's instructions (Invitrogen). Quantitative real-time PCR was performed for all samples using following primers designed by the software primer premier 6.0. Primers for human leptin (Accession No. NM_000230.2), anti-SMA mRNA were normalized with 18S and 

Western Blotting of p-JAK-1/p-STAT3 and α-SMA Cells were centrifuged and lysed in gel loading buffer (500 mM Tris–HCl, pH 6.8, 5% sodium dodecyl sulfate (SDS), 20% glycerol, 50 μl bromophenol blue). Forty micrograms of total cell lysate were boiled, separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10%) and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked with 5% bovine serum albumin (BSA)/0.05% Tween 20 in phosphate buffer saline (PBS) (PBS-T) at room temperature, incubated with anti-p-JAK-1 (Cell Signaling Technology, Inc. (CST)), anti-JAK-1 (CST), anti-p-STAT3 (CST), anti-STAT3 (CST) and anti-α-SMA rabbit polyclonal antibody (Abcam), washed 3 times with PBS-T, and then incubated with goat anti-rabbit immunoglobulin G (IgG)–HRP conjugate antibody (Molecular Probes, U.S.A.). The signal was finally detected using the enhanced chemiluminescence substrate Super Signal West Dura (Pierce, U.S.A.). As loading control, the membrane was stripped and hybridized with anti-β-actin (Abcam) antibody. Quantification of band intensity was performed with Glyco Band-Scan software.

Enzyme-Linked Immunosorbent Assay (ELISA) Leptin (DLP00, R&D, U.S.A.) and collagen I (CSB-E13445h, Cusbio Biotech Co., Ltd., China) concentration in culture media were measured by the standard sandwich ELISA following manufacturer’s instructions. The cells (2×10^3 cells/well) were cultured in 96-well plate for 24 h, and then transfected with miRNA and/or incubated with TMP for 48 h. Cell culture supernates were collected and stored at −80°C for future assay. A total of 6 parallel cells were set for each group. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad, U.S.A.).

Statistical Analysis Data are calculated as the arithmetic means (mean) and standard error (S.E.), and statistical analysis was performed with paired Student’s t-test. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

The Fibrosis Activity of Cells Was Discrepant in Differ-

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**Fig. 1.** The Fibrotic Phenotype of Cells Was Observed by the Light Microscope

(A) Control group, without any treatment; (B) hsa-miR-9 group, transfection of hsa-miR-9; (C) anti-hsa-miR-9 group, transfection of anti-hsa-miR-9; (D) TMP group, treatment with TMP (200 mg/L); (E) combined treatment group, transfection of hsa-miR-9 and treatment with TMP (200 mg/L). Original magnification, ×40.
The activated HSCs in control group exhibited a stretched morphology with developed stress fibers. hsa-miR-9-Transfected suppressed cell activity, and similar results were obtained by TMP treatment and combined treatment. However, anti-hsa-miR-9-transfected group show same morphology as control (Fig. 1).

**hsa-miR-9 Was Differentially Expressed in Control and Treated Groups** Assay of the expression level of hsa-miR-9 in each treatment group by quantitative real-time PCR demonstrated that miRNA of hsa-miR-9 was respectively up-regulated by 2.5, 2.0 and 3.1-fold in the hsa-miR-9-transfected group, TMP-treated group, combination treated group compared with that of control group. But in anti-hsa-miR-9-transfected group, the miRNA was decreased by 0.3-fold (Student’s *t*-test, *p*<0.05). Moreover, the miR-9 in the combining treated group showed obviously higher expression than those in the other groups (Student’s *t*-test, *p*<0.05) (Fig. 2). These results suggest that the endogenous levels of hsa-miR-9 were enhanced or inhibited by transfection of sense or anti-sense strand hsa-miR-9.

**Combination Treatment Group with hsa-miR-9 and TMP Inhibited Leptin Expression Significantly** To ex-
explore the relationship between miR-9 and leptin, the mRNA of leptin in each group was assayed by quantitative real-time PCR. As a result, the mRNA showed the highest expression in the anti-hsa-miR-9-transfected group among the 5 groups, followed by the control group, TMP-treated group, and hsa-miR-9-transfected group. But in the combination treatment group, the expression of leptin was the lowest. Except for the anti-hsa-miR-9-transfected group, the levels of mRNA in other groups were significantly lower than the control group (Student’s t-test, \( p<0.05 \)). In addition, the hsa-miR-9-transfected group and TMP-treated group demonstrated a higher expression of mRNA than that of combination treatment group (Fig. 3A). To further analyze the protein level of leptin, ELISA was carried out and the concentrations of supernatant in each group were calculated as 96.30±6.03, 53.14±2.13, 99.30±8.11, 47.14±4.11 and 42.79±2.84, respectively, suggesting that the expression trend of leptin on protein level was consistent with those on their mRNA levels (Fig. 3B). In addition, hsa-miR-9 and leptin were subjected to an assay by online analysis (www.targetscan.org), and hsa-miR-9 was predicted to bind with mRNA of leptin thus induce the degradation of the gene (Fig. 4). This likely explains the opposite expression model of leptin to hsa-miR-9.

**hsa-miR-9 and TMP Combination Treatment Group Inhibited HSC Activation by Blocking the JAK1/STAT3 Pathway** To evaluate the effect of hsa-miR-9 and TMP on the downstream signal pathway of leptin, phosphorylation of JAK1/STAT3 in each group was investigated by Western blotting. As expected, the highest level of p-JAK1 (30.25±1.25) and p-STAT3 (25.36±2.33) were detected in the anti-hsa-miR-9-transfected group, followed by the control group (22.12±1.58 and 15.18±0.97) and hsa-miR-9 transfection group (20.22±1.38 and 16.25±1.56) and combination treatment group (15.30±1.36 and 17.25±1.05) (Fig. 5), the phosphorylation trend of which is consistent with the expression model of leptin. This suggests that hsa-miR-9 and TMP might hinder the activation of LX-2 cell by blocking the JAK1/STAT3 signal pathway.

**\( \alpha \)-SMA and Collagen I Levels Were Significantly Decreased in hsa-miR-9 and TMP Combined Treatment Group** Since \( \alpha \)-SMA is an indicator of activated HSC, the mRNA level of \( \alpha \)-SMA was down-regulated 5.34-fold, 7.4-fold, and 10-fold in the hsa-miR-9, TMP and combined treatment group compared with control group, while level in the anti-hsa-miR-9 group was up-regulated 1.2-fold (Fig. 6A). The protein levels of \( \alpha \)-SMA showed the similar trends with that of mRNA levels (Fig. 6B), the expression of \( \alpha \)-SMA was the lowest in the combination treatment group (9.79±2.24), while it was the highest in the anti-hsa-miR-9-transfected group (29.30±4.11) among the 5 groups. The Anti-hsa-miR-9-transfected group expressed higher \( \alpha \)-SMA than that in the hsa-miR-9 transfection group (13.13±2.25) and TMP-treated group (12.38±3.62), which proved that hsa-miR-9 and TMP can collaboratively down-regulate the expression of \( \alpha \)-SMA through the JAK1/STAT3 signal pathway, thus inhibiting the development of HSC activation.

The ELISA results demonstrated that the changes in collagen I showed no difference statistically between the hsa-miR-9 transfection group (27.42±2.71) and control group (29.51±4.27), while the relatively higher expression of collagen I was detected in the anti-hsa-miR-9-transfected group (32.01±5.34). However, the expression of collagen I was obviously down-regulated in the combination treatment group (15.71±2.84) and TMP-treated group (20.14±2.02) (Fig. 7).
DISCUSSION

HSC plays important roles in the occurrence and development of liver fibrosis. When liver is damaged by harmful factors, HSC is converted to myofibroblasts through a series of activation processes and leads to liver fibrosis. Therefore, HSC is considered as a suitable target for studying the mechanisms and development of medicine for liver fibrosis. The cell line LX-2 employed in the study is proved to be such an activated cell, which are widely applied in practices.\(^7\)

In this study, we focused on the effects of hsa-miR-9 on leptin expression. Over-expression or interference of hsa-miR-9 in LX-2, has directly resulted in the down-regulation or up-regulation of leptin. Bioinformatics analysis showed that hsa-miR-9 could interact with the 3’UTR of leptin. These results fully suggested that hsa-miR-9 could be involved in the post-transcriptional regulation of leptin, thus affecting the development of liver fibrosis. These results are consistent with previous reports on the regulation mechanisms of other miRNAs.\(^8-10\)

TMP is an alkaloid monomer contained in the traditional Chinese medicine in Chuanxiong, which is known to promote blood circulation by removing blood stasis.\(^4\) In the present study, TMP treatment could effectively increase the expression of hsa-miR-9 compared with control group. Our previous research on animals have indicated that TMP could inhibit the expression level of leptin to hinder the development of hepatic fibrosis. Thus we speculate that TMP might block leptin-mediated JAK/STAT and TGF-β1/Smads two different signal pathways to hinder the development of fibrosis.

CONCLUSION

Our results demonstrated that hsa-miR-9 transfection, TMP treatment or a treatment of both TMP and hsa-miR-9 transfection could effectively lower the leptin expression at mRNA and protein levels compared with that of the control group. Furthermore, the levels of phosphorylated JAK1/STAT3, \(\alpha\)-SMA and collagen I were apparently down-regulated in the above groups. We think that TMP more likely increase the level of the hsa-miR-9 to degrade the expression of leptin gene, preventing from the development of liver fibrosis through the JAK1/STAT3 signal pathway, which would provide the promising thoughts for the disease.

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Conflict of Interest The authors declare no conflict of interest.

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Fig. 7. The Concentration of Collagen I in Different Group by ELISA

The concentration of collagen I in culture media was measured by ELISA. All groups were treated as shown in Materials and Methods. Control group without any treatment, hsa-miR-9 group and anti-hsa-miR-9 group were subjected to the transfection of hsa-miR-9 and anti-hsa-miR-9 separately. TMP group was treated with TMP (200 mg/L), combined treatment group was transferred with hsa-miR-9 and treated with TMP (200 mg/L). Results were presented as the mean±S.E. *\(p<0.05\), **\(p<0.01\) vs. control; *\(p<0.05\), **\(p<0.01\) vs. combined treatment group (hsa-miR-9 and TMP).

