Inhibition of miR-182 represses growth of hepatocellular carcinoma cells by targeting SOX11

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Abstract

Purpose: To investigate the effect of microRNA-182 (miR-182) on hepatocellular carcinoma (HCC) and its mechanisms of action.

Methods: Sixty-five HCC tissues and matched adjacent tissues were obtained, and miR-182 and sex-determining region on the Y Chromosome (SRY)-related HMG box 11 (SOX11) expression were quantified using reverse transcription-polymerase chain reaction (RT-PCR). SOX11-positive cells were identified by immunohistochemistry and the correlation between SOX11 and miR-182 expression was analyzed by Spearman correlation analysis. Huh-7 cells were transfected with the miR-182 mimic, the miR-182 inhibitor, and/or si-SOX11, and cell proliferation was measured by cell counting kit-8 (CCK-8) assay. Apoptosis was assessed by flow cytometry. Luciferase reporter assay was used to investigate the putative target gene of miR-182. A xenograft nude mouse model was established by transfection with miR-182 antagonir, while tumor volume and weight were calculated. SOX11, cleaved caspase-3, cleaved poly (ADP ribose) polymerase (PARP), B-cell lymphoma-2 (Bcl-2), and Bcl-2-associated X (Bax) protein levels were analyzed by western blot.

Results: MiR-182 expression increased and SOX11 expression was decreased in HCC tissues (p < 0.05). Luciferase reporter assay data confirmed that miR-182 directly targets SOX11. Inhibition of miR-182 repressed proliferation and Bcl-2 expression, but increased protein expression of cleaved caspase-3, cleaved RAPP, and Bax in Huh-7 cells (p < 0.05). In addition, suppression of SOX11 reversed the effects of miR-182 on cell proliferation and apoptosis. The miR-182 antagonist decreased tumor growth and miR-182 expression but increased SOX11 expression in vivo (p < 0.05).

Conclusion: MiR-182 and SOX11 may be novel therapeutic targets for HCC patients.

Keywords: microRNA-182, Hepatocellular carcinoma, Apoptosis, SOX11

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth-most common cancer and the third leading cause of cancer-related death [1]. HCC accounts for ~90% of primary liver cancers, and > 50% of the cases worldwide are in China [1]. Many therapeutic strategies have been implemented in
the clinical treatment of liver cancer, including drug intervention, chemo-embolization, conservative treatment, and surgical treatment. Recently, new cancer therapeutic targets have been identified for various cancers [2]. However, HCC treatment remains a challenge due to its inherent chemoresistance, pharmacologic challenges caused by the decompensated liver, difficulty in accurate radiological response assessment, and the uncertain survival benefit of chemotherapy.

MicroRNAs (miRNAs) are small non-coding RNAs that play key roles in tumorigenesis by negatively regulating mRNA expression of target genes. Compelling evidence has shown that dysregulated miR-182 expression functions in the biological processes of multiple cancer types. A recent study demonstrated a high level of miR-182 expression in the human gastrointestinal stromal tumor (GIST) and that overexpression of miR-182 promoted GIST cell proliferation, suggesting that an miR-182 antagonist may be a potential treatment for GIST [3]. It was also shown that miR-182 increased the cisplatin resistance of HCC cells [4]. Further, miR-182 was shown to attenuate liver cancer by augmenting natural killer cell cytotoxicity [5]. However, the mechanism of action of miR-182 in HCC is still poorly understood.

SOX11 is an essential member of the SOX family of transcription factors, and it functions as an important regulator of embryogenesis and carcinogenesis and as a tumor suppressor. SOX11 is considered an oncogene, and its expression is frequently increased in various cancers, including epithelial ovarian cancer. However, expression of SOX11 was downregulated in HCC, and SOX11 overexpression inhibited growth and induced apoptosis by regulating the Wingless and INT-1 (Wnt)/β-catenin pathways in Huh-7 cells [6]. However, whether miR-182 targets SOX11 to regulate proliferation and apoptosis of HCC remains unclear.

Thus, this study investigated the effect of miR-182 on HCC and the underlying mechanism.

EXPERIMENTAL

Tissues

Sixty-five HCC tissues and matched adjacent tissues were obtained from Affiliated Hospital of North Sichuan Medical College and were fixed with 4 % paraformaldehyde and embedded in paraffin. Three μm thick sections were prepared for further experiments. All procedures involving human participants were in accordance with standards upheld by the Ethics Committee of Affiliated Hospital of North Sichuan Medical College, and approved accordingly, and followed the 1964 Helsinki Declaration and its later amendments for ethical research involving human subjects [7].

Cell culture

The human HCC Huh-7 cell line was purchased from the Institute of Life Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco, USA) supplemented with 10 % heat-inactivated fetal bovine serum, 0.6 mg/mL L-glutamate, and 1× penicillin/streptomycin in a 37°C incubator under 5% CO₂/95% air. The cells were passaged for four generations prior to experimental use.

Cell transfection

The miR-182 mimic was purchased from Qiagen (Germany) and was used to overexpress hsa-miR-182. Huh-7 cells were transfected with the miR-182 mimic (Qiagen) or the mimic control (Qiagen). The miR-182 inhibitor was used to inhibit hsa-miR-182, and an inhibitor control was also used. For cell transfection, 2.5 × 10⁵ Huh-7 cells were placed in the wells of 6-well plates. A 5 nM miRNA solution was made by diluting 150 ng of miRNA in 100 mL serum-free medium. Transfections of Huh-7 cells with miRNAs were conducted using HiPerFect Transfection Reagent (Qiagen), according to the manufacturer’s instructions. The transfected cells were cultured under normal growth conditions.

A non-specific scramble small interfering RNA (siRNA) was used as the negative control (NC). The siRNA against SOX11 and the NC siRNA were purchased from Invitrogen (Invitrogen, USA). When the Huh-7 cells reached ~70% confluency, 2.5 × 10⁵ cells were transfected with 200 pmol target siRNA or NC siRNA using 10 μL of Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. After incubation for 44 h, the cells were collected for further experiments. All experiments were performed at least in triplicate. miRNA and siRNA sequences are listed in Table S1.

Luciferase reporter assay

TargetScan (release 7.2, http://www.targetscan.org/) was used to identify potential target genes. The 3'-untranslated region (UTR) of SOX11 was cloned into the psicheck2 plasmid. The mutated miRNA seed sequence for SOX11
was also cloned into psicheck2. Huh-7 cells were co-transfected with psicheck2-SOX11-wt or psicheck2-SOX11-mut and the miR-182 mimic or the NC mimic. A dual-reporter luciferase assay system (Switchgear Genomics) was used to measure luciferase activity.

Table S1: miRNA and siRNA sequences

| Gene   | Sequences (5'-3') |
|--------|------------------|
| miR-182 mimic | UUUGGCAAUGUGAAGACACACU |
| mimc control | ACAUCUGCGUAAGAUUGACU |
| miR-182 inhibitor | AGUGUGAGUUCCACUUGCCAA |
| inhibitor control | CAGUACUUUUGUGUAGUACAA |
| SOX11 siRNA | CACUCUAACGUGUCAUGU |
| negative control | UUGUACUACACAAAGGUACUG |

Cell proliferation assay

Huh-7 cells (2 x 10^4 cells/well) were maintained in a 48-well plate, and cell proliferation was evaluated using the Cell Counting Assay Kit-8 (CCK-8, #CK04, Dojindo, Japan) according to the manufacturer's instructions. A microplate reader (Sigma-Aldrich, USA) was used to measure absorbance at 450 nm.

Apoptosis assay

Cells transfected with the miR-182 mimic, the miR-182 inhibitor, or the mimic control were incubated for 72 h and then washed twice with cold PBS. The cells were then stained with propidium iodide and fluorescein isothiocyanate (FITC)-labeled Annexin V and analyzed by flow cytometry.

Xenograft model establishment

The Lenti-Pac 293Ta packaging cell line was transfected with lentivirus expression plasmids using the Lenti-Pac HIV Expression Packaging Kit. Then, lentivirus was transfected with the hsa-miR-182 antagonist vector or the NC antagonist and incubated for 14 h at 37°C. Huh-7 cells were incubated in medium containing 1/500 volume of TiterBoost reagent for 72 h, and then the Huh-7 cells were infected with lentiviral particles using 8 µg/mL Polybrene (Sigma-Aldrich). Hygromycin (100 µg/mL, Invitrogen) was used to select cells that were stably transfected for 21 days. A total of 12 BALB/c-nu/nu mice (4-week-old) were obtained from Charles River Laboratory Animal Co., Ltd (USA). Animals were maintained according to the Guide for the Care and Use of Laboratory Animals [8], and this study was approved by the Institutional Animal Care and Use Committee of Affiliated Hospital of North Sichuan Medical College. Huh-7 cells (1 x 10^7) stably expressing the miR-182 antagonist or the NC antagonist were injected subcutaneously into the right front side flanks of 6 BALB/c nu/nu mice each. Tumor sizes were measured with calipers twice per week for 6 weeks. Tumor volume (V) was determined using Eq 1.

V = x^2y/2(x < y) \ldots (1)

Quantitative real-time RT-PCR

RNA was extracted from xenograft tissues using TRizol reagent (Invitrogen). RNA was also obtained from HCC and adjacent tissues using the mirNeasy FFPE kit (#217504, Qiagen). RNA concentrations were estimated by their A260/A280 ratios [9,10]. Complementary DNA (cDNA) was synthesized using the RevertAid First Strand cDNA Synthesis kit (#K1622, Thermo Scientific, USA).

Table 1: Primer sequences for amplification

| Variable | Gene   | Forward | Reverse |
|----------|--------|---------|---------|
| NC_000072.7 | mmu_miR-182 | 5'-CATGGGATATCGGGGCTT | 5'-GTACCGGCAGCGGATGCATAGAC |
| NC_00007.14 | has_miR-182 | 5'-UUUGGCAAUGUGAAGACACU | 5'-UGUGAGUUCUACUUGCCAA |
| NC_000078.7 | mmu_SOX11 | 5'-ACCCGGACTGTGCAAGAC | 5'-CGACTGCTCCATGCTTTCTT |
| NC_0000002.12 | has_SOX11 | 5'-CATGGGATATCGGGGCTT | 5'-GTACCGGCAGCGGATGCATAGAC |
| NC_000072.7 | mmu_GAPDH | 5'-ATGGTGCTCGTGAGATTTG | 5'-ATGGCGGCTTCCACCTCCT |
| NC_000012.12 | has_GAPDH | 5'-CAATGACCCCTCATTGACCC | 5'-TGTTTTGGAGGATCTCAG |
| NC_000083.7 | mmu_U6 | 5'-GACGGGAGACAGAGATGG | 5'-AAAGCCCTTCGAGATCACG |
| NC_000015.10 | mmu_U6 | 5'-GCTTGCCAGCAGCATATAC | 5'-GTGCCGGTGCCGAGATTC |
Quantitative real-time RT-PCR was carried out with the Maxima SYBR Green qPCR Master Mix Kit (#K0252, Thermo Scientific) [11], and an Applied Biotechnology 7500 Real-time PCR system version 3.0. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were used as internal controls. The 2^−ΔΔCT method was used to quantify relative RNA expression [12]. The primer sequences used for amplification are shown in Table 1.

**Immunohistochemical assay**

Paraffin-embedded specimens were used to construct a tissue microarray using a manual tissue arrayer (MTA-1, Beecher Inc, USA). Then, 3 µm sections of tissue microarrays were prepared and incubated with rabbit anti-SOX11 antibody (#ab242203, 1:500, Abcam, UK) at room temperature for 25 min. SOX11 was stained using an antigen retrieval method in which sections were incubated in 0.1 M Tris-HCl at 90°C for 10 min. Signals were detected with the Dako REAL Detection system, and sections were counterstained with Mayers haematoxylin (Sigma-Aldrich). A Leica fluorescent microscope with an attached CCD camera (Wetzlar, Germany) was used to obtain images.

**Western blot analysis**

Huh-7 cells and tissues were digested in radio-immunoprecipitation assay (RIPA) lysis buffer (Invitrogen), and then proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk and then incubated with primary monoclonal antibodies against SOX11 (#ab170916, 1:1000, Abcam), cleaved caspase-3 (#ab32042, 1:500, Abcam), cleaved PARP (#ab32064, 1:1000, Abcam), Bax (#BS1030, 1:250, Bioworld, USA), Bcl-2 (#4223, 1:100, Cell Signaling Technology, USA), or GAPDH (#ab8245, 1:1000, Abcam) overnight. Then, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:10000, Abcam). Blots were visualized using a PhyLight™ enhanced chemiluminescence kit (#PH0353, Phygene Scientific, China), and band intensities were normalized to the GAPDH control.

**Statistical analysis**

All statistical analyses were performed with SPSS 18.0 software (USA). Data were expressed as mean ± standard deviation. Differences between two groups were analyzed using the t-test. Differences between multiple groups were analyzed by one-way analysis of variance followed by Tukey’s multiple comparison test. Spearman correlation analysis was used to evaluate the relationship between miR-182 and SOX11 in HCC tissues. $P < 0.05$ was considered statistically significant.

**RESULTS**

**Increased miR-182 expression and decreased SOX11 expression in HCC tissues**

miR-182 expression was higher in HCC tissues than in adjacent tissues ($p < 0.001$; Figure 1A). SOX11 expression was downregulated in HCC tissues and the number of SOX11-positive cells was lower in HCC tissues than in adjacent tissues ($p < 0.001$; Figures 1B and 1C). Western blot analysis showed that SOX11 protein expression was downregulated in HCC tissues when compared with adjacent tissues ($p < 0.001$; Figure 1D).

Spearman correlation analysis showed a negative correlation between SOX11 expression and miR-182 expression ($r = -0.2686$, $P = 0.0305$; Figure 1E). These findings suggest that miR-182 expression was increased and Sox11 expression was decreased in HCC tissues.
SOX11 is a target gene of miR-182

It was predicted that the AACGGUU sequence in miR-182 pairs with the UUGCCAA sequence in the 3′-UTR of SOX11 (Figure 2A). The luciferase reporter assay showed that overexpression of miR-182 decreased the luciferase activity of SOX11-wt when compared with the NC mimic group (p < 0.001; Figure 2B). There was no significant difference between the luciferase activity of the SOX11-mut and the miR-182 mimic and NC mimic (Figure 2B). Western blot analysis showed that overexpression of miR-182 decreased SOX11 protein expression when compared with the NC mimic (p < 0.001), whereas the miR-182 inhibitor increased SOX11 protein expression when compared with the NC inhibitor (p < 0.001; Figure 2C). These findings suggest that SOX11 is a target gene of miR-182.

Figure 2: miR-182 targets SOX11 directly. (A) The predicted pairing of the SOX target region and miR-182. (B) Luciferase activity was measured after transfection with miR-182. (C) After transfection with miR-182 or miR-182 inhibitor, SOX11 protein levels were evaluated by western blot analysis. ***P < 0.001, miR-182 mimic vs NC mimic or miR-182 inhibitor vs NC inhibitor.

Inhibition of miR-182 suppresses proliferation and promotes apoptosis of Huh-7 cells

The CCK-8 assay was used to determine the effect of miR-182 on Huh-7 cell proliferation 72 h after transfection. Overexpression of miR-182 increased cell proliferation when compared with the NC mimic group (p < 0.001). Cell proliferation was visibly repressed 72 h after transfection with the miR-182 inhibitor when compared with the NC inhibitor group (p < 0.001; Figure 3A). Overexpression of miR-182 markedly inhibited apoptosis of Huh-7 cells when compared with the NC mimic (p < 0.01), and the miR-182 inhibitor promoted Huh-7 cell apoptosis (p < 0.001; Figure 3B). Western blot analysis showed that overexpression of miR-182 suppressed cleaved caspase-3, cleaved PARP, and Bax protein levels but increased the protein level of Bcl-2 when compared with the NC mimic (p < 0.001). Inhibition of miR-182 increased the protein levels of cleaved caspase-3, cleaved PARP, and Bax but decreased the protein level of Bcl-2 when compared with the NC inhibitor (p < 0.001; Figure 3C). These results showed that inhibition of miR-182 suppressed cell proliferation and promoted apoptosis of Huh-7 cells.

Figure 3: Inhibition of miR-182 represses cell proliferation and promotes apoptosis of Huh-7 cells. (A) After 72 h of transfection with the miR-182 mimic or the miR-182 inhibitor, Huh-7 cell proliferation was assessed using the CCK-8 assay. (B) After transfection with the miR-182 mimic or the miR-182 inhibitor, Huh-7 cell apoptosis was assessed by flow cytometry. (C) After transfection with the miR-182 mimic or the miR-182 inhibitor, protein levels of cleaved caspase-3, cleaved PARP, Bax, and Bcl-2 were analyzed by western blot analysis. ***P < 0.001 and **P < 0.01, miR-182 mimic vs NC mimic or miR-182 inhibitor vs NC inhibitor.

Inhibition of SOX11 reverses the effect of miR-182

To determine whether inhibition of SOX11 would alter the effect of the miR-182 inhibitor on Huh-7 cell proliferation and apoptosis, SOX11 was knocked down with siRNA. The miR-182 inhibitor suppressed cell proliferation compared with NC inhibitor (p < 0.001; Figure 4A). Upon knockdown of SOX11, cell proliferation increased with the miR-182 inhibitor or the NC inhibitor (p < 0.001). Flow cytometry showed that the miR-182 inhibitor promoted cell apoptosis when compared with the NC inhibitor (p < 0.001). However, inhibition of SOX11 repressed cell apoptosis with the NC inhibitor or the miR-182 inhibitor (p < 0.001; Figure 4B). These data suggested that inhibition of SOX11 reverses the effect of miR-182 on Huh-7 cell proliferation and apoptosis.
Inhibition of SOX11 reverses the effect of miR-182 on Huh-7 cell proliferation and apoptosis. (A) Huh-7 cell proliferation was assessed by CCK-8 assay 72 h after transfection with si-SOX11 and/or miR-182 inhibitor. (B) Huh-7 cell apoptosis was analyzed by flow cytometry after transfection with si-SOX11 and/or miR-182 inhibitor. ***p < 0.001, si-NC + NC inhibitor vs si-NC + miR-182 inhibitor or si-SOX11 + NC inhibitor; or si-NC + miR-182 inhibitor vs si-SOX11 + miR-182 inhibitor

Inhibition of miR-182 represses tumor growth in vivo

A cell-derived xenograft mouse model was established by transfection with the miR-182 antagonist. The miR-182 antagonist decreased the gross tumor volume and tumor weight when compared with the NC antagonim (p < 0.001; Figure 5A and 5B). Expression of miR-182 decreased upon inhibition of miR-182 (p < 0.001), whereas SOX11 expression increased (p < 0.001; Figure 5C). The SOX11 protein level also increased upon inhibition of miR-182 (p < 0.001; Figure 5D). These results revealed that inhibition of miR-182 represses tumor growth in vivo.

DISCUSSION

HCC is a complex disease with complex pathophysiology [1]. Increasing evidence shows that miRNAs play critical roles in cancer development, progression, and metastasis, and miRNAs are potential biomarkers for cancer prognosis. Therefore, it is necessary to investigate the role of miRNAs in HCC and explore their target genes. The results of this study indicated that miR-182 exerts an inhibitory effect on HCC by directly targeting SOX11 and may be a potential therapeutic target for the treatment of HCC.

Aberrant expression of miRNAs has been implicated in the progression of HCC. miRNA-21 functions as an oncogene and is frequently overexpressed in HCC. Aberrant miR-21 expression is related to HCC cell proliferation, migration, invasion, and tumor growth [13]. In addition, it was shown that upregulation of miR-106b and miR-93 increased HCC cell apoptosis by regulating the Bcl-2 interacting mediator (Bim) of cell death [14].

MiRNA-182 was shown to be significantly elevated in HCC tissues and to directly target the CCAAT enhancer binding protein alpha (Cebpa), indicating that it contributes to HCC progression and may be used as a therapeutic drug target or diagnostic marker for HCC [15]. miR-96 and miR-182 were also elevated in HCC, and inhibition of miR-96 and miR-182 repressed HCC cell proliferation and invasion via the Eph receptor interacting protein (ephrin) A5 [16]. The results from this study are consistent with these previous findings. In HCC tissues, miR-182 expression was increased and overexpression of miR-182 promoted cell proliferation and decreased apoptosis of Huh-7 cells, whereas inhibition of miR-182 repressed cell proliferation and induced apoptosis. These data suggested that abnormal miR-182 expression may contribute to HCC and that inhibition of miR-182 suppresses cell proliferation and promotes apoptosis of Huh-7 cells.

Recently, it has been shown that SOX11 is dysregulated in various cancers. In mantle cell
lymphoma, SOX11 was downregulated in cells that overexpressed miR-223, and the luciferase reporter assay showed that miR-223 inhibits the wild-type 3'-UTR of SOX11 and not the mutant 3'-UTR of SOX11. In addition, miR-223 expression correlated negatively with SOX11 expression. Further, inhibition of miR-223 associated with poor clinical outcomes for mantle cell lymphoma. It has been shown that maternally expressed gene 3 (MEG3) and SOX11 levels were decreased and the miR-9-5p level was increased in HCC and that overexpression of MEG3 induced HCC cell apoptosis and suppressed HCC cell growth by sponging miR-9-5p, which directly targets SOX11 [17]. In this study, SOX11 was decreased in HCC tissues, and the luciferase reporter assay showed that miR-182 inhibits the wild-type 3'-UTR of SOX11. In addition, inhibition of SOX11 reversed the effect of miR-182 on Huh-7 cell proliferation and apoptosis. These findings indicate that inhibition of miR-182 represses growth of HCC cells by directly targeting SOX11.

**CONCLUSION**

Although this study revealed that miR-182 exerts an inhibitory effect on HCC by directly targeting SOX11, clinical experiments are needed to confirm these findings. In addition, an in-depth understanding of miR-182 targeting of SOX11 is also required. Thus, the findings of this study demonstrate that miR-182 inhibits HCC cell growth by directly targeting SOX11, and this may lead to the development of a new therapeutic approach for the management of HCC patients.

**DECLARATIONS**

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**Conflict of interest**

No conflict of interest is associated with this study.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Contribution of authors**

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zhi Liu and Jianyu Chen designed the study and supervised the data collection. Lin Mo analyzed and interpreted the data. Chuan Lan, Hui Chen, Jianshui Li, and Songlin Hou prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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