Up-regulated miR-500a enhances hepatocarcinoma metastasis by repressing PTEN expression

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Introduction

Hepatocarcinoma is a common and aggressive cancer that is strongly associated with chronic infection by the hepatitis B virus (HBV) [1]. Poor prognosis and patient survival with hepatocarcinoma are largely due to invasion/metastasis and postsurgical recurrence [2]. Invasion and metastasis are fundamental properties of hepatocellular carcinoma (HCC), which has a very high mortality rate. Metastasis is a complex cascade, however, and the underlying molecular mechanisms are far from being fully understood [3].

MiRNAs are a class of endogenous phylogenetically conserved small RNAs (~22 nts) responsible for the post-transcriptional regulation of mRNA translation and stability. They are involved in several biological processes, such as development, apoptosis, proliferation, and differentiation. aberrant expression of numerous miRNAs has been associated with cancer development [4], and deregulated miRNAs have been linked to molecular pathways involved in neoplastic transformation [5]. Altered expression of miRNAs and the identification of their molecular target genes in hepatocarcinoma have previously been described by our and other groups [6,7]. Therefore, taking advantage of promising in vivo studies on miRNAs, this class of molecules may represent a new kind of unconventional targeted treatment to be eventually associated with traditional approaches for hepatocarcinoma not amenable of curative therapies [8]. As previous study showed miR-500a was found as an oncofetal miRNA in liver cancer.
increased amount of miR-500a was found in the sera of the HCC patients [9]. But the detailed mechanism of miR-500a regulating liver cancer is unknown.

The aim of the present study was to investigate the relationship between miRNAs and the proliferation and metastasis of hepatocarcinoma and the related molecular mechanism. First, we determined the level of miR-500a in serum and tissues from metastatic and non-metastatic hepatocarcinoma patients. Then, hepatocarcinoma cells were transfected with mimic miR-500a or inhibitor miR-500a, and the proliferation capacity and invasion of cells were determined. Moreover, we found that miR-500a influenced AKT signaling pathway by targeting PTEN.

Materials and methods

Serum and tissue samples

Serum samples were obtained from 12 patients with hepatocarcinoma and 17 cases of the normal people to study and compare the expression of miR-500a. Moreover, serum and tissues were also obtained from ten cases of non-metastatic hepatocarcinoma patients and seven cases of metastatic hepatocarcinoma patients. All patients underwent surgery in the Huaihe Hospital of Henan University (Henan, China) during February 2013 to November 2015. Before surgery, there had not undergone chemotherapy or radiotherapy.

Peripheral blood was obtained in the early morning from all individuals. Sera were separated by centrifuging the blood samples at 3000 rpm for 15 min. The samples were stored at −80°C or fixed in formaldehyde after resection. All patients’ samples were obtained at their first visit. Clinical and laboratory data reported in the present study were obtained at the time of sampling. The present study was approved by Ethics Committee of Huaihe Hospital of Henan University and written informed consents were obtained before the patients and healthy volunteers entered into the present study.

RNA isolation

Total RNA was isolated from fresh hepatocarcinoma tissue and serum samples using the miRNeasy Mini Kit (Qiagen, Valencia, CA, U.S.A.) and miRVana RNA isolation kit (Ambion Inc, Austin, TX, U.S.A.) according to the manufacturer’s protocol. RNA purity and concentrations were measured with the Nanodrop 2000 (Thermo Fisher Scientific, San Jose, CA, U.S.A.). RNA integrity was detected on an agarose gel with Ethidium Bromide staining by electrophoresis. The RNA samples were immediately stored at −80°C for next cDNA conversion.

Cell culture

Human hepatocarcinoma cell lines SMMC-7721 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were kept in RPMI-1640 medium (Gibco, U.S.A) in humidified air containing 5% CO₂ at 37°C. RPMI-1640 medium contained 10% FBS (HyClone, U.S.A.) and 1% penicillin/streptomycin.

Cell transfection

Cells were seeded in six-well plate and cultured overnight. MiR-500a mimics, corresponding negative control (mimic NC), the siRNAs targetting miR-500a (inhibitor miR-500a) and corresponding negative control (inhibitor NC) were synthesized and purified by GenePharma (Shanghai, China). MiR-500a-overexpressed plasmid (pCDNA3.1-miR-500a) and blank vector pCDNA3.1 were purchased from Chinese Academy of Sciences (Changchun, China). Cells were transfected with the oligonucleotide using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions.

qRT-PCR

Total mRNA was extracted from the retinal samples with TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s protocol. Pipette was used to beat upon retinal samples evenly and samples were put into 1.5-ml Eppendorf tubes to stand for 5 min to separate nucleic acid–protein complex. Each EP tube was added with 1 ml TRIzol and 200 μl precooled chloroform. The tubes were shook for 15 s and centrifuged at 4°C, 12000 rpm for 15 min. We drew aqueous phase from other 1.5-ml EP tubes and added 0.5-ml isopropanol. The mixture was mixed and centrifuged at 4°C, 12000 rpm for 15 min. After removing the supernatant, 1 ml precooled 75% ethanol was added to wash RNA precipitate and this procedure was repeated for three times. Then, the RNA precipitate was dried in vacuum and concentration was determined with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, U.S.A.). PCR tubes were prepared, 1 μl oligo dT (0.5 μg/μl) and 2 μg total RNA were put into each tube. After that, diethylyrocarbonate (DEPC) was added into each PCR tube to volume of 12 μl. After mixing evenly, tubes were placed in 65°C water-bath kettle for 5 min and then placed on ice immediately.
Reverse transcription was performed at 55°C for 30 min, initial activation for 15 min at 95°C, next 40 cycles of denaturation were conducted at 94°C for 15 s, then annealed for 30 s at 55°C, extension for 30 s at 72°C. The expression level was normalized using U6 snRNA by the 2-ΔΔCt method. The ΔCt values were normalized to U6 level. Precursor miRNA (pre-miRNA) or the mature miRNA were used as a template for qRT-PCR. The sequence of U6 was RT of AACCCTTCAGAATTTGGC, F of CTCGCTTCGGGACACA and R of AACGTTCCAGA ATTGGCGT. The sequence of mir-500 was RT of GTCGTATCCAGTGCAGGGTTCG AGGTATTCCACTGAGATACACTCTGAC, F of TATAAACCTTGGTACCTGG and R of GTGCAGGGTCCAGG. The sequence of cel-miR-39-3p was RT of GTCGTATCCAGTGCAGGGTTCG ACTGAGAGACAGCAGGCT, F of TTATCACGGGTGTA AAC and R of GTGCAGGGTCCAGG.

Western blotting
The main function of PTEN is to negatively regulate PI3K-AKT signaling pathway, which can activate the AKT and its downstream gene p-Akt (S473), p-Akt (T308). Akt, p-mTOR, mTOR, p-4E-BP1, 4E-BP1, p-S6K, and S6K through phosphorylation to promote cell proliferation, migration, and anti-apoptosis. Therefore, we used Western blotting to determine the protein expression of p-Akt (S473), p-Akt (T308), Akt, p-mTOR, mTOR, p-4E-BP1, 4E-BP1, p-S6K, and S6K in cells after transfection with mimic miR-500a or inhibitor miR-500a. Total proteins from the cells were extracted by ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with 1 mM protease inhibitor PMSF (Sigma, St. Louis, MO, U.S.A.). The protein concentration was quantitated with a BCA assay kit (Beyotime, Shanghai, China). Equal amounts of protein were separated by SDS/PAGE (10% gel), transferred on to PVDF (Millipore, Bedford, MA, U.S.A.) membrane, and then blocked with 5% non-fat milk in TBS. The membranes were incubated with primary antibodies, mouse anti-human monoclonal PTEN, Akt, mTOR, 4E-BP1, and S6K antibody (Santa Cruz Biotechnology, CA, U.S.A.) and mouse anti-human monoclonal β-actin antibody (Santa Cruz Biotechnology, CA, U.S.A.), at 4°C overnight. The membranes were washed and subsequently probed with secondary antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at a 1/4000 dilution for 1 h at room temperature. Proteins were visualized with chemiluminescent detection system (ECL; Beyotime). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control.

Cell viability assay
Cells were seeded in the 96-well plate for 24 h after transfected at a density of 1500 cells/well. The cell viability assay was performed using Cell Counting Kit-8 (CCK8; Dojindo) according to the manufacturer’s protocol every 2 days. The absorbance at 450 nm was measured. Experiments were performed thrice.

Cell invasion assay
Cell invasion assay was performed using 24-well transwell chambers containing 8-mm pore diameter polycarbonate membrane (Corning, New York, NY, U.S.A.). Two hundred microliters of cell suspension containing 4 × 10^4 cells were added into the upper chamber, and 500 μl of culture medium containing 10% (v/v) FBS was added into the lower chamber. After incubation at 37°C under 5% (v/v) CO2 for 24 h, the non-filtered cells were gently removed with a cotton swab, and the migrated cells were fixed with 100% methanol, stained with 0.5% Crystal Violet and washed with PBS (Gibco). The invaded cell number was counted under the microscope.

Luciferase activity assays
Wild-type and mutant 3'-UTR of PTEN sequences were cloned into the psi-mediated instrumental response (pMIR) luciferase reporter vector (Thermo Fisher, U.S.A.). For the luciferase assays, 100 ng pMIR luciferase reporter vector was co-transfected in cells with 100 nM miR-500a mimics or control regent, together with 20 ng pMIR-REPORT β-gal Control Plasmid (Thermo Fisher, U.S.A.) as an internal normalized control. Cells were harvested 48 h after transfection and the luciferase activities were assayed according to the manufacturer’s protocol. Transfections were performed in duplicate and repeated three times.

Clone formation
Cell suspension (5 × 10^6 cells/ml) of SMMC-7721 was seeded into two 60-mm Petri dishes with 2 × 10^2 cells in each dish. The cells were dispersed evenly by slightly shaking the Petri dishes and were then incubated at 37°C with 5% CO2 for 21 days until the visible clones appeared. The medium was discarded and the cells were carefully washed with PBS twice. After being fixed with methanol for 15 min, the cells were stained with Giemsa’s solution for 15 min before washing with tap water and air drying. The clones with more than 50 cells were counted with an ordinary
Overexpression of miR-500a promoted the proliferation and invasion of hepatocarcinoma

To investigate the effect of miR-500a in hepatocarcinoma cell proliferation, SMMC-7721 cells were treated with mimic miR-500a and inhibitor miR-500a for 2, 4, and 6 days. qRT-PCR was used to verify the transfection effect. Results showed that the expression level of miR-500a increased significantly after transfection with mimic miR-500a (Figure 2A) and decreased observably after transfection with inhibitor miR-500a (Figure 2C). Cell proliferation assay revealed that relative SMMC-7721 cell number increased significantly after transfection with mimic miR-500a for 4 and 6 days (Figure 2B), and decreased dramatically after transfection with inhibitor miR-500a for 2, 4, and 6 days (Figure 2D). Moreover, a transwell assay showed that overexpression of miR-500a significantly promoted the invasion of SMMC-7721 cells compared with the cells transfected with mimic NC (Figure 2E,F), while knockdown of miR-500a inhibited the invasion of cells compared with the cells transfected with inhibitor NC (Figure 2G,H).
miR-500a, w h i n e t e a x p r e s s i o n o f p (h a d a s i g n i f i c a n t d i f f e r e n c e . ( ) c e l l n u m b e r i n m i c k e n e a s u r e d b y a t r a n s w e l l a s s a y m e t h o d a f t e r t r a n s f e c t i o n f o r 4 8 h. **P<0.01, compared with the NC group, the relative or average cell number in mimic miR-500a or inhibitor miR-500a group had significant difference.

**Figure 2. MiR-500a regulated the proliferation in SMMC-7721 cells**

(A,B) Transfection of mimic miR-500a increased miR-500a level, while inhibitor miR-500a decreased the level. RNA expression was measured by real-time PCR. *P<0.05 and **P<0.01, compared with mimic miR-500a group, the relative cell number in mimic NC group had a significant difference. (C,D) Transfection with mimic miR-500a accelerated SMMC-7721 cell proliferation, while inhibitor miR-500a inhibited cell proliferation. Cell proliferation was measured by CCK8 kit in after transfection with mimic or inhibitor miR-500 for 0, 2, 4, and 6 days. *P<0.05 and **P<0.01, compared with inhibitor miR-500a group, the relative cell number in inhibitor NC group had a significant difference. (E,F) Cell invasion ability was increased when transfected with mimic miR-500a in SMMC-7721 cells. (G,H) Cell invasion ability was decreased when transfected with inhibitor miR-500a in SMMC-7721 cells. Cell invasion abilities were measured by a transwell assay method after transfection for 48 h. **P<0.01, compared with the NC group, the relative or average cell number in mimic miR-500a or inhibitor miR-500a group had significant difference.

**Overexpression of miR-500a increased the cloning of hepatocarcinoma cells**

As the above results showed, we obtained that the overexpression of miR-500a promoted the proliferation capacity of hepatocarcinoma cells. To further explore the influence, we cloned SMMC-7721 cells after transfection with mimic miR-500a and inhibitor miR-500a. Results showed that the clone number of SMMC-7721 cells was significantly increased after transfection with mimic miR-500a compared with the mimic NC group and decreased after transfection with inhibitor miR-500a compared with the inhibitor NC group (Figure 3A–D).

**PTEN is a direct target of miR-500a in hepatocarcinoma**

By target gene prediction analysis, we found that there had been binding sites between PTEN and miRNA. To explore whether PTEN was a direct target for miR-500a, we constructed the 3'-UTR reporter plasmids coupled with full length of PTEN 3'-UTR with wild-type (wt) or mutant (mut) miR-500-binding sites (Figure 4A). Luciferase assay showed that miR-500a could repress the expression of reporter gene containing wt 3'-UTR but not that containing mut 3'-UTR (Figure 4B). Then, we determined the protein expression values of PTEN, p-Akt (S473), p-Akt (T308), Akt, p-mTOR, mTOR, p-4E-BP1, 4E-BP1, p-S6K, and S6K in SMMC-7721 cells after transfection with mimic NC, mimic miR-500a, inhibitor NC, and inhibitor miR-500a. Results showed that the protein expression of PTEN was significantly decreased after transfection with mimic miR-500a, while the expression was increased observably after transfection with inhibitor miR-500a (Figure 4C,D). The protein expression values of p-Akt (S473)/Akt, p-mTOR/mTOR, and p-4E-BP1/4E-BP1 were increased observably after transfection with mimic miR-500a, while the expression was significantly decreased after transfection with inhibitor miR-500a (Figure 4E,G,H). The protein expression of p-Akt (T308)/Akt and p-S6K/S6K were decreased observably after transfection with inhibitor miR-500a, while transfection of mimic miR-500a had no influence on the expression values (Figure 4F,I).
Figure 3. MiR-500a regulated clone formation in SMMC-7721 cells
(A) Clone formation was increased when transfected with mimic miR-500a in SMMC-7721 cells. (B) The column diagram about the average clone number in mimic NC and mimic miR-500a group. **P<0.01, compared with the mimic NC group, the average clone number of SMMC-7721 cells in mimic miR-500a had a significant difference. (C) Clone formation was decreased when transfected with inhibitor miR-500a in SMMC-7721 cells. (D) The column diagram about the average clone number in inhibitor NC and inhibitor miR-500a group. *P<0.05, compared with the inhibitor NC group, the average clone number of SMMC-7721 cells in inhibitor miR-500a had a significant difference.

Discussion

Hepatocarcinoma is a common visceral malignancy and amongst the leading causes of cancer deaths worldwide. It typically arises in a setting of chronic hepatitis or cirrhosis, with infection by hepatitis B and C viruses and chronic exposure to aflatoxin B together responsible for ~80% of all hepatocarcinoma cases in humans [10]. miRNAs are a class of small noncoding RNAs that control gene expression by targetting mRNAs and triggering either translation repression or RNA degradation [11]. They have rapidly emerged as modulators of gene expression in cancer in which they may have great diagnostic and therapeutic importance [12]. MiR-500a is a key player in breast cancer survival and the expression of miR-500a is increased in highly tumorigenic derivative of MDA-MB-231 cell line compared with its low tumorigenic parental cell line [13]. Moreover, median levels of miR-500a were higher in HCC patients than in healthy controls [14].

Our study showed that the pre-miRNA level of serum miR-500a in hepatocarcinoma patients was significantly higher than the normal. The expression of serum miR-500a was significantly increased in metastatic hepatocarcinoma patients than non-metastatic ones. The level of miR-500a in hepatocarcinoma tumor tissues was dramatically increased compared with the adjacent tissues. It indicated that miR-500a was up-regulated in patients with hepatocarcinoma, which manifested that the expression of miR-500a was closely related with hepatocarcinoma. MiR-500a-3p was found to play a role in breast cancer proliferation as well as doxorubicin-induced cardiotoxicity [15]. To further explore the role of miR-500a on hepatocarcinoma, we transfected hepatocarcinoma cell lines SMMC-7721 with mimic miR-500a or inhibitor miR-500a, and then determined the proliferation, invasion, and cloning capacity of SMMC-7721 cells. Results showed that overexpression of miR-500a increased the proliferation, invasion, and cloning capacity of SMMC-7721 cells, while knockdown of miR-500a decreased the capacity. It further verified the role of miR-500a on hepatocarcinoma.
PTEN can be used clinically to suppress tumor and inhibit the activation of PI3K/AKT signaling pathway [16]. The loss of PTEN can result in the activation of AKT kinases, which play key roles in cell growth, proliferation, and invasion [17]. Here, we identified PTEN as a direct and functional target of miR-500a. Study showed miR-500a overexpression inhibited the expression of PTEN. The protein expression values of PTEN, p-Akt (S473), p-Akt (T308), Akt, p-mTOR, mTOR, p-4E-BP1, 4E-BP1, p-S6K, and S6K in SMMC-7721 cells after transfection with mimic miR-500a or inhibitor miR-500a. (D–I) The relative optical density of PTEN, p-Akt (S473), p-Akt (T308), p-mTOR/mTOR, p-4E-BP1/4E-BP1, and p-S6K/S6K in SMMC-7721 cells after transfection with mimic miR-500a or inhibitor miR-500a. *P<0.05 and **P<0.01, compared with the NC group, the relative optical density in mimic miR-500a or inhibitor miR-500a group had a significant difference.

Competing interests
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Author contribution
Y.Z., Y.h.W., and Y.q.W. designed and conducted experiments. Y.Z. and Y.h.W. surveyed literature and developed text of the manuscript. Y.h.W. helped in statistical analysis. Y.Z. and Y.h.W. refined the write up. Y.q.W. revised the paper accordingly. All authors read and approved the final manuscript.

Abbreviations
HCC, hepatocellular carcinoma; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; pMIR, psi-mediated instrumental response; pre-miRNA, precursor miRNA; PTEN, phosphatase and tensin homologue; qRT-PCR, quantitative reverse transcription-PCR; RIPA, radioimmunoprecipitation assay.

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