**miR-1297 promotes apoptosis and inhibits the proliferation and invasion of hepatocellular carcinoma cells by targeting HMGA2**

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**Abstract.** MicroRNAs (miRNAs) have recently emerged as important regulators of gene expression in various tissues. In particular, miRNAs have been identified as new therapeutic agents and biomarkers in cancer. The aim of the present study was to explore whether miR-1297 has an anti-cancer role in hepatocellular carcinoma cell lines and to explore its underlying mechanism. The proliferation, apoptosis and migration of hepatocellular carcinoma cells were evaluated by cell viability assay, TUNEL staining and a wound healing assay, respectively. Western blot analysis and reverse transcription polymerase chain reaction (RT-PCR) were performed to determine the expression levels of proteins and mRNAs of high-mobility group AT-hook 2 (HMGA2) in hepatocellular carcinoma. The luciferase assay was employed to verify the inhibitory activity of miR-1297 on the 3' untranslated region (3'UTR) of the HMGA2 gene. In the present study, overexpression of miR-1297 significantly inhibited the proliferation of HepG2 and SMMC7721 cells. Forced expression of miR-1297 also increased the apoptosis of HepG2 and SMMC7721. Furthermore, the migration of HepG2 and SMMC7721 was also clearly suppressed by miR-1297 overexpression. All these effects can be abrogated by co-transfection with miR-1297 inhibitor-AMO-1297. The luciferase assay verified that miR-1297 overexpression is able to inhibit the activity of luciferase reporter harboring the HMGA2 3'UTR, indicating HMGA2 as the target of miR-1297. Although the HMGA2 level was not affected by miR-1297, the HMGA2 protein was significantly inhibited by miR-1297 overexpression. Collectively, miR-1297 was revealed to regulate the proliferation, apoptosis and migration of hepatocellular carcinoma cells via acting on HMGA2. The finding provides a new target for the treatment of hepatocellular carcinoma.

**Introduction**

Hepatocellular carcinoma remains the most common type of liver cancer, and usually develops secondary to cirrhosis or viral hepatitis infection. The incidence of hepatocellular carcinoma has gradually increased in certain developed and developing countries. Despite advances in multidisciplinary therapies, the median survival time of patients with hepatocellular carcinoma is ~9 months (1). Therefore, the characterization of molecular mechanisms underlying the initiation and progress of hepatocellular carcinoma is necessary. Certain factors, including inflammation, oxidative stress and hypoxia, have been shown to facilitate the initiation, progression and metastasis of hepatocellular carcinoma (2). The androgen receptor has been reported to regulate cell adhesion and migration of hepatocellular carcinoma via regulating the β1-integrin-AKT signaling pathway (3). Overexpression of actopaxin may lead to the increase of invasion and migration ability, and an epithelial-mesenchymal transition process in hepatocellular carcinoma (4). mTOR signaling was also shown to have a critical role in the pathogenesis of hepatocellular carcinoma (5). Oxysterol-binding protein (OSBP)-related protein 8 (ORP8) markedly inhibited tumor growth and induced cell apoptosis of hepatocellular carcinoma cells through the Fas/FasL pathway in vivo and in vitro (6).

MicroRNAs (miRNA) have been recognized as potential therapeutic targets for hepatocellular carcinoma. miRNAs are a class of small non-coding RNA molecules and post-transcriptionally regulate protein expression by targeting the 3' untranslated region (3'UTR) of target mRNA, causing degradation or repression of translation (7). Previous studies have reported that miRNAs have important roles in the development and maintenance of various diseases, such as cancer, heart hypertrophy and fibrosis (8-11). For example, in hearts, the upregulation of miR-23a induced by isoproterenol or aldosterone caused hypertrophic growth of cardiomyocytes, and the knockdown of miR-23a could attenuate cardiomyocyte hypertrophy by regulating NFATc3 (12). Particularly in cancer, miRNAs have emerged as new potential therapeutic molecules.
and targets (13,14). The miR-543 level was significantly reduced in hepatocellular carcinoma, and overexpression of miR-543 enhanced the proliferation and invasion of HepG2 via targeting PAQR3 in hepatocellular carcinoma (13). Additionally, miR-520e can inhibit the growth of hepatocellular carcinoma cells, and the knockdown of miR-520e lead to increased cell proliferation (14). Overexpression of miR-375 reduced cell proliferation and migration, and also induced G1 arrest and apoptosis in hepatocellular carcinoma cells via targeting at AEG-1 (15). miR-1 has been shown to promote apoptosis of HepG2 by targeting apoptosis inhibitor-5 (16). It was previously reported that miR-1297 exerted antitumor effects on human lung adenocarcinoma cell and colorectal cancer (17,18), and acted as a tumor suppressor. However, whether miR-1297 has an inhibitory effect on hepatocellular carcinoma has not been determined.

To the best of our knowledge, the present study established for the first time, a potential association between miR-1297 and the proliferation, apoptosis and migration of hepatocellular carcinoma cells. The antitumor effects of miR-1297 on HepG2 and SMMC7721 cells were investigated, and the precise mechanism of miR-1297 was explored in inhibiting liver cancer.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from HyClone Laboratories. Lipofectamine™ 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Hsa-miR-1297 mimics (miR-1297), hsa-miR-1297 inhibitor (AMO-1297) and its scramble negative control (control) were synthesized by Sangon (Shanghai, China). Unless otherwise specified, all the other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture. Human hepatocellular carcinoma HepG2 and SMMC7721 cell lines were purchased from the China Center for Type Culture Collection (Wuhan, China). These two cell lines were cultured in DMEM supplemented with 10% FBS and penicillin (100 U/ml)/streptomycin (100 µg/ml), and were subsequently transferred into plastic culture flasks and cultured at 37˚C in humid air with 5% CO₂.

miRNAs transfection. miRNAs were transfected into HepG2 and SMMC7721 at 50-70% confluence using Lipofectamine 2000 reagent according to the manufacturer's instructions. The final oligonucleotide concentration of miR-1297, AMO-1297 or its scramble reached 50 nmol/l. Three hours after miRNAs transfection, the culture medium was changed to fresh DMEM with 10% FBS. Subsequently, HepG2 and SMMC7721 were incubated for an additional 48 h and harvested for further investigation. These transfected oligonucleotides were divided into four groups: Control, hsa-miR-1297 mimics (miR-1297), hsa-miR-1297 + hsa-miR-1297 inhibitor (miR-1297 + AMO-1297) and its scramble negative control (scramble).

Cell viability assay. Cell viability of HepG2 and SMMC7721 was determined by the MTT assay, according to the methods described in a previous study (14). In brief, HepG2 and SMMC7721 cells were seeded in 96-well plates, and were transfected with miR-1297, miR-1297 + AMO-1297 or its scramble. After transfection, the cells were exposed to 10 µl of MTT solution (5 mg/ml) for 2-4 h at 37˚C. After the supernatant was removed from formazan crystals, and dimethyl sulfoxide (DMSO) was added. The cell viability was calculated according to the absorbance at 570 nm using an OPTImax microplate reader.

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) assay. Apoptosis of HepG2 and SMMC7721 cells was determined using a TUNEL detection kit (Roche, Penzberg, Germany) according to the manufacturer's instructions. After transfection with miR-1297, miR-1297 + AMO-1297 or its scramble, HepG2 and SMMC7721 cells were fixed with 4% paraformaldehyde in PBS for 1 h at room temperature, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Following washing in PBS, sections were incubated with the TUNEL reaction mixture for 1 h at 37˚C. After washing with PBS, the stained cells were visualized using a fluorescence microscope (Eclipse TE300; Nikon, Tokyo, Japan).

Wound healing assay. HepG2 and SMMC7721 cells were cultured in a 6-well plate for 24 h. The wound healing assay was carried out by introducing a small linear scratch with a pipette tip. Subsequently, the cells in each well were incubated with serum-free DMEM medium. Images of 48 h following the scratch in cultured cells were captured under a phase-contrast microscope (magnification, x200) to monitor the cell migration process.

Transwell assay. The Transwell assay was performed using a Transwell chamber with pore size of 8.0 µm (Millipore, Billerica, MA, USA). The cells were resuspended in serum-free DMEM medium and were placed in the upper chamber in 5% CO₂ at 37˚C. Following transfection, cultured HepG2 and SMMC7721 cells in the upper chamber were removed, and the attached cells in the lower section were stained with 0.1% crystal violet. The migration rate was quantified by counting the migration cells in six random fields under a light microscope.

Bioinformatics prediction. The potential target gene of miR-1297 was predicted by computer-aided algorithms using TargetScan (http://www.targetscan.org).

Luciferase reporter assay. After plating in a 24-well plate, HEK293 cells were transfected with the constructed reporter plasmid containing 3'UTR of high-mobility group AT-hook 2 (HMG A2) plus miR-1297, miR-1297 + AMO-1297 or its scramble. Luciferase assays were performed using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA) 24 h after transfection.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from the cells using TRIzol according to the manufacturer's instructions. RT-qPCR was conducted using an RT-PCR kit (Takara, Dalian, China). The primers used were...
all synthesized by Sangon. Semi-quantitative RT-PCR was performed using a thermal cycler (Bio-Rad, Hercules, CA, USA). The primer sequences for human HMGA2 gene expression were as follows: HMGA2 forward, 5'-TGGAGGAGA GCGAAATCTAAA-3' and reverse, 5'-AAGACCTTG GTCAACCACATC-3'; and β-actin forward, 5'-TGAAGATCACA GATCATCTGCTCC-3' and reverse, 5'-GCCATGCCAAT CTCATCTTG-3'. The relative HMGA2 mRNA levels were normalized to those of β-actin mRNA levels using Quality One analysis software (Bio-Rad).

Western blot analysis. The protein samples were extracted from the cultured HepG2 and SMMC7721 cells. After 12,000 x g centrifugation at 4°C for 10 min, the concentration of extracted protein was determined by the bicinchoninic acid method (Beyotime, Jiangsu, China). The extracted proteins were separated using 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently electrothermally transferred to the PVDF membrane (Millipore). The membranes were blocked in blocking buffer (5% non-fat milk dissolved in PBS-Tween-20) overnight at 4°C. Subsequently, the membranes were incubated with goat polyclonal antibodies against HMGA2 (sc-23684) and mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (sc-32233; Santa Cruz Biotechnology, Inc.) and detected by ECL Detection Systems (Thermo Scientific, Waltham, MA, USA). The GAPDH protein was used as an internal control.

Statistical analysis. Statistical data are shown as mean ± standard error of the mean. All the statistical analysis was performed by SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Differences between two groups were defined with a t-test, and the significant differences among three groups were determined using analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-1297 reduces the cellular viability of HepG2 and SMMC7721 cells. To evaluate the effects of miR-1297 on the proliferation of HepG2 and SMMC7721 cells, the MTT assay was used to measure the cellular viability of HepG2 and SMMC7721. The overexpression of miR-1297 resulted in a significant reduction of cellular viability in HepG2 cells compared with the control (P<0.05) (Fig. 1A). Co-transfection with AMO-1297 may abolish the inhibitory effects of miR-1297 on the viability of HepG2 cells (P<0.05) (Fig. 1A). The scrambled negative control did not significantly induce the apoptosis of HepG2 cells. A similar anti-proliferation action of miR-1297 was also observed in SMMC7721 cells (Fig. 1B). These results suggest that miR-1297 has an inhibitory role in the proliferation of HepG2 and SMMC7721 cells.

miR-1297 induces the apoptosis of HepG2 and SMMC7721 cells. Whether miR-1297 overexpression was able to induce apoptosis of HepG2 and SMMC7721 cells was further studied. The TUNEL assay was carried out to investigate the influence of miR-1297 on the apoptosis of HepG2 and SMMC7721 cells. Fig. 2A demonstrates that miR-1297 overexpression induced a significant increase of HepG2 cells that were positive for TUNEL staining compared with the control (P<0.05). Similarly, the number of HepG2 cells positive for TUNEL staining was also increased by miR-1297 overexpression in SMMC7721 cells (P<0.05) (Fig. 2C). Co-transfection with AMO-1297 may suppress the increase of apoptosis by miR-1297 in HepG2 and SMMC7721 cells (Fig. 2). The scrambled negative control did not significantly induce the apoptosis of HepG2 and SMMC7721 cells.

miR-1297 inhibits the migration of HepG2 and SMMC7721 cells. In addition to cellular proliferation, the effect of miR-1297 on the migration activity of HepG2 and SMMC7721 cells was examined. The wound healing assay in vitro was carried out to determine cell migration of HepG2 and SMMC7721. Fig. 3A shows the quantified wound closure in cultured HepG2 cells following miR-1297, co-transfection with AMO-1297 and its scramble transfection. Wound closure was significantly slowed in miR-1297-treated HepG2 and SMMC7721 cells at 48 h after the scratch, compared with the control group (Fig. 3B and C). Consistently, the Transwell migration assay was also performed in HepG2 and SMMC7721 cells with miR-1297, co-transfection with AMO-1297 and its scramble transfection. In agreement with the wound healing assay, transfection with miR-1297 overexpression produced
Figure 2. Effect of miR-1297 on the apoptosis of HepG2 and SMMC7721 cells. (A) The representative images of terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL)-positive HepG2 cells transfected with miR-1297, miR-1297 + AMO-1297 and its scramble. (B) Statistical analysis of apoptotic HepG2 cells following transfection of miR-1297, miR-1297 + AMO-1297 and its scramble. (C) The representative images of TUNEL-positive SMMC7721 cells following miR-1297, miR-1297 + AMO-1297 and its scramble transfection. (D) miR-1297 induced the increase of apoptotic SMMC7721 cells, and co-transfection with AMO-1297 abolished this change. Data are expressed as mean ± standard error of the mean. N=5. *P<0.05 vs. control; #P<0.05 vs. miR-1297.

Figure 3. Effect of miR-1297 on the migration of HepG2 and SMMC7721 cells by the wound healing assay. (A) Representative images of HepG2 cells of four groups following the scratch. (B) Statistical analysis of wound width of HepG2 cells with the transfection of miR-1297, miR-1297 + AMO-1297 and its scramble. (C) Wound healing assay showed the inhibitory effects of miR-1297 on the migration of SMMC7721 cells. Data are expressed as mean ± standard error of the mean. N=3. *P<0.05 vs. control; #P<0.05 vs. miR-1297.
an inhibition of migration of HepG2 (Fig. 4A and B) and SMMC7721 cells (Fig. 4C and D). This data demonstrated that the suppression of migration of hepatocellular carcinoma cells was induced by miR-1297.

miR-1297 targets the 3'UTR of HMGA2. A bioinformatics database of miRNAs was used (http://www.targetscan.org/) to predict the potential target gene of miR-1297. miR-1297 was shown to have a putative binding site to the 3'UTR region of HMGA2 (Fig. 5A). A highly conservative miR-1297 binding site at the HMGA2 3'UTR 1867-1873 base position was predicted in humans. Subsequently, the Dual-Luciferase reporter assay was performed to confirm that miR-1297 directly targeted the 3'UTR of HMGA2. Overexpression of miR-1297 decreased the luciferase activity of the reporter plasmid harboring HMGA2 3'UTR, suggesting that miR-1297 inhibits the translation of HMGA2 by binding to its 3'UTR (Fig. 5B). However, co-transfection of AMO-1297 alleviated the reduction in luciferase activity caused by miR-1297 in HEK293. These results indicate that HMGA2 is a direct target of miR-1297.

miR-1297 regulates HMGA2 expression in hepatocellular carcinoma. Regulatory effects of miR-1297 on HMGA2 expression were further confirmed. miR-1297, miR-1297 + AMO-1297 or its scramble were transfected into HepG2 and SMMC7721 cells, and western blotting was subsequently used to determine the level of HMGA2 protein. Fig. 6 demonstrates that the overexpression of miR-1297 caused a significant reduction of HMGA2 protein in HepG2 and SMMC7721 cells compared with control (P<0.05). By contrast, co-transfection with AMO-1297 significantly reversed the reduction of the HMGA2 protein following miR-1297 transfection (P<0.05). Transfection of scramble sequence did not affect the expression of HMGA2 protein in HepG2 and SMMC7721.
cells. This finding indicates that miR-1297 inhibits HMGA2 protein expression in hepatocellular carcinoma.

Discussion

In the present study, miR-1297 inhibited the proliferation and migration, and promoted the apoptosis of hepatocellular carcinoma, and the direct target of miR-1297 is the HMGA2 gene. This finding provides a new therapeutic approach for liver cancer.

Hepatocellular carcinoma is the most common type of liver cancer, accounting for ~90% liver cancers (19). Currently, hepatocellular carcinoma has become the third leading cause of cancer fatalities worldwide (19). Numerous studies have revealed that liver cirrhosis and viral hepatitis infection are two important risk factors of hepatocellular carcinoma (20). In addition, inflammation, necrosis, fibrosis and ongoing regeneration also contribute to cirrhotic liver and lead to the development of hepatocellular carcinoma (2). Recent studies have characterized the altered molecular pathways, such as
p53, PIKCA and β-catenin genes during carcinogenesis, and the mutations on these genes was most frequently reported in patients with hepatocellular carcinoma (21-24). The abnormal activation of Wnt/β-catenin appears to be frequently altered in hepatocellular carcinoma (23,24). mTOR signaling was also shown to have a critical role in the pathogenesis of hepatocellular carcinoma (5). Androgen receptor has been reported to regulate cell adhesion and migration of hepatocellular carcinoma via regulating the β1-integrin-AKT signaling pathway (3). Recently, miRNAs have been recognized as a potential therapeutic target for hepatocellular carcinoma. In the present study, the molecular mechanism of antitumor activity of miR-1297 on hepatocellular carcinoma was explored.

To investigate whether miR-1297 has an antitumor role in HepG2 and SMMC7721, the MTT assay was employed to observe the effects of miR-1297 on cellular viability of HepG2 and SMMC7721. The results showed that miR-1297 can significantly inhibit the proliferation of HepG2 and SMMC7721 cells, and co-transfection with AMO-1297 can abolish the inhibition of cell viability in HepG2 and SMMC7721 cells. Additionally, the effects of miR-1297 overexpression were assessed on the apoptosis of HepG2 and SMMC7721, and the results showed that forced expression of miR-1297 may lead to the apoptosis of HepG2 and SMMC7721. In agreement with the present findings, previous studies also reported that miR-1297 inhibited the growth of human lung adenocarcinoma cell and colorectal cancer (17,18). COX2 and TRIB2 were identified as the target genes of miR-1297 in two types of cells, and have been shown to be associated with the inhibition of proliferation. However, whether COX2 and TRIB2 are involved in the miR-1297 inhibition of liver cancer cell proliferation remains unknown. It has been reported that TRIB2 contributes to the inhibition of proliferation and the induction of apoptosis of HepG2 cells (25). It is thus proposed that TRIB2 is a possible target of miR-1297 in hepatocellular carcinoma cells.

Migration of cancer cells is an important factor for cancer metastasis. Inhibition of migration contributed to the treatment of hepatocellular carcinoma (3). The impact of miR-1297 overexpression on the migration of hepatocellular carcinoma was also observed in the present study. Overexpression of miR-1297 significantly inhibited the migration of HepG2 and SMMC7721 cells, and co-transfection with AMO-1297 may attenuate the slowed migration of hepatocellular carcinoma by miR-1297. Consistently, it has been reported that miR-1297 inhibited the migration and invasion of human colorectal cancer in vivo and in vitro (17).

Bioinformatics predication on a microRNA database (TargetScan) was used to screen the possible candidate gene of miR-1297. A binding site of miR-1297 was identified in the 3’UTR of HMG2A, suggesting HMG2A as a potential target of miR-1297. Activation of the HMG2A gene was observed in the cells of numerous human malignancies, such as breast cancer, lung cancer and pancreatic carcinoma (26-30). The inhibition or silencing of HMG2A contributed to the inhibition of tumor growth, the induction of apoptosis and the suppression of tumor metastasis (31). In the present study, the luciferase assay showed that miR-1297 overexpression markedly inhibited the luciferase activity of the reporter plasmid harboring the 3’UTR of HMG2A gene. To the best of our knowledge, this is the first study to uncover the inhibition effects of miR-1297 on HMG2A in hepatocellular carcinoma cells.

To further confirm that miR-1297 overexpression inhibits the translation of HMG2A in hepatocellular carcinoma, the effects of miR-1297 were studied on the expression of the HMG2A protein in HepG2 and SMMC7721. Overexpression of miR-1297 also reduced the expression of HMG2A protein in the HepG2 and SMMC7721 cells. Whereas, the mRNA level of HMG2A was not affected by overexpression of miR-1297. These results suggest that miR-1297 directly acts on the 3’UTR of HMG2A gene.

In conclusion, this is the first study to show that miR-1297 inhibited the proliferation and migration, and promoted the apoptosis of hepatocellular carcinoma cells via directly targeting the HMG2A gene. miR-1297 is a potential therapeutic target of hepatocellular carcinoma.

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References