

MicroRNA-497 regulates cell proliferation in hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver. MicroRNA-497 (miR-497) is known to be downregulated in several types of human cancer; however, the expression, function and underlying mechanisms of miR-497 in HCC remain unclear. Therefore, the present study investigated miR-497 expression in HCC samples and HCC-derived cell lines using reverse transcription-quantitative polymerase chain reaction. The protein expression of one of the predicted common targets of miR-497, insulin-like growth factor-1 receptor (IGF-1R), was assessed using western blot analyses and immunohistochemistry. The role of miR-497 in regulating the proliferation of HCC-derived cells was also investigated *in vitro* and *in vivo*. Of 60 paired specimens from HCC patients, miR-497 was downregulated in 42 cancer specimens compared with adjacent non-cancer tissues. Western blotting and immunohistochemical analyses revealed that IGF-1R expression was significantly increased in HCC compared to control tissues. In addition, overexpression of miR-497 was observed to inhibit colony formation and tumor growth in MHCC-97H human HCC cells. Conversely, SMMC-7721 human HCC cells transfected with a miR-497 inhibitor exhibited enhanced colony formation and tumor growth. Finally, IGF-1R protein, phosphoinositide 3-kinase/Akt signaling pathway-associated proteins and cyclin pathway-associated proteins were differentially expressed between miR-497-overexpressing cells and

miR-497-silenced cells. These results indicate that miR-497 may be a potentially effective gene therapy target.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the third most common cause of cancer-associated mortality worldwide, with >600,000 mortalities reported annually (1,2). Over the last few decades, the incidence of HCC has increased in eastern Asia and sub-Saharan Africa (3,4); the estimated number of new cases diagnosed annually increased from 437,000 to 564,000 between 1990 and 2000 (1,4). Due to the highly aggressive nature of the tumor, and because tumors are highly resistant to traditional treatments, such as chemotherapy and radiation, the 5-year survival rate of HCC is poor, with an overall survival rate of <16% (5). In addition, surgical resection and liver transplantation are restricted, and are suitable only for patients diagnosed with early stage disease (6). It is generally recognized that exploring the underlying molecular mechanisms of HCC initiation and progression in order to search for functional molecular targets may provide a new approach for HCC treatment.

MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs, which act as key post-transcriptional regulators of target-gene expression. They bind primarily to 3'-untranslated regions (3'-UTRs) of target gene mRNAs. This process leads to translational repression or mRNA cleavage (7,8). Recent studies have reported that >1,000 human miRNAs have been identified that regulate ~1/3 of the coding genes in the human genome (9). Many of these miRNAs act as tumor suppressors and/or oncogenes, and are involved in cell activities that include development, differentiation, proliferation, apoptosis, metabolism and immunity (7,10,11). Recently, an increasing number of studies have revealed that microRNA-497 (miR-497) levels are decreased in tumors, and that it functions as a tumor suppressor in a number of types of human cancer, including colorectal, gastric, cervical and breast cancers, adrenocortical carcinoma and melanoma (12-20). Similarly, a study by Furuta *et al* (21) indicated that miR-497

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targets multiple cell cycle regulators and suppresses cell cycle progression *in vitro*. However, whether miR-497 regulates other target genes in HCC is unknown.

Insulin-like growth factor-1 receptor (IGF-1R) is a member of the receptor tyrosine kinase family and contains two extracellular α subunits (including the ligand-binding site) and two β subunits (with intracellular tyrosine kinase activity) (22,23). IGF-1R has an important role in malignant cell growth and survival, and is highly expressed in malignant tumors in the nervous system, liver, thymus, adrenal cortex, gallbladder, colon, pancreas and lung (24-28). Furthermore, IGF-1R is crucial for activating the phosphoinositide 3-kinase (PI3K)/Akt pathway (29,30), which promotes cell proliferation and survival, and is activated by numerous growth factor receptor tyrosine kinases (31-33). IGF-1R mRNA and protein expression are increased in HCC and are closely associated with the progression of malignant tumors (34,35). To date, studies have demonstrated that miR-497 targets IGF-1R and has a tumor suppressive role in human cervical cancer (20) and in colorectal cancer (12). However, whether miR-497 functions as a tumor suppressor by directly targeting IGF-1R in HCC remains unclear.

In the current study, the expression levels of miR-497 and IGF-1R were examined in HCC cells and tumor samples. miR-497 overexpression was found to inhibit cell growth, reduce IGF-1R expression and decrease PI3K/Akt pathway activation. Although downregulation of miR-497 contributed to malignant behavior in HCC cells, it increased IGF-1R expression and elevated activation of PI3K/Akt signaling. These results suggest that miR-497 functions as tumor suppressor by targeting IGF-1R in HCC.

Materials and methods

Ethics statement. The Institutional Animal Care and Use Committee at Nanjing Medical University (Nanjing, China) approved the study protocols for experiments involving human tissue and animals.

Tissue specimens. A total of 60 paired HCC and adjacent non-tumor tissues were evaluated for the expression of miR-497 and IGF-1R. Adjacent non-tumor tissues were ≥ 2 cm away from the edge of the tumors. All tissues were obtained from patients that had undergone partial hepatectomy at the First Affiliated Hospital of Nanjing Medical University between December 2011 and February 2014. The patient cohort included 49 males and 11 females, with a median age of 58 years (range, 36-66 years). Of the 60 patients, 18 patients exhibited stage A disease, 28 patients exhibited stage B and 14 patients exhibited stage C disease, according to the Barcelona Clinic Liver Cancer staging system (36). A total of 35 cases exhibited an HCC mass >5 cm in diameter, while 25 cases exhibited an HCC mass with a diameter of <5 cm. Patients that had undergone treatment prior to surgery were excluded from the study. HCC specimens and adjacent non-tumor tissues were confirmed by pathological examination, and immediately stored in liquid nitrogen post surgery. Written informed consent was obtained from either the patient or the families of the patients.

Liver cancer cell lines. Human HCC cell lines (YY-8103, HepG2, Hep3B, SMMC-7721 and MHCC-97H) and normal human

liver cells (L02) were obtained from the Department of Liver Transplantation Center at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (both HyClone™; GE Healthcare Life Sciences, Logan, UT, USA), penicillin (50 U/ml; Invitrogen™; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) and streptomycin (50 μ g/ml; Invitrogen™; Thermo Fisher Scientific, Inc.) and propagated in 5% CO₂ in a 37°C humidified incubator.

Reverse transcription (RT)-quantitative polymerase chain reaction (qPCR). Total RNA from liver tissue samples and HCC cell lines were extracted using Invitrogen™ TRIzol reagent (Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. To determine miRNA expression, total RNA (1 μ l/sample) was reverse-transcribed using miRNA-specific stem-loop RT primers, reverse transcriptase, RT buffer, dNTPs and an RNase inhibitor, according to the manufacturer's instructions (TaqMan® MicroRNA Reverse Transcription Kit; Applied Biosystems™; Thermo Fisher Scientific, Inc.). qPCR was performed using an Applied Biosystems™ StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Inc.). The 20- μ l reaction system contained the corresponding cDNA (2 μ l), miRNA-specific TaqMan® primers (1 μ l), TaqMan® Universal PCR Master Mix (10 μ l) and ddH₂O (7 μ l) (Applied Biosystems™; Thermo Fisher Scientific, Inc.). The PCR conditions were 95°C for 10 min, followed by 50 cycles at 95°C for 15 sec and 60°C for 1 min. RNU6B was used as an endogenous housekeeping control for data normalization of miRNA levels. The comparative threshold cycle (Ct) method was used to measure the relative changes in expression (37); $2^{-\Delta\Delta C_t}$ represents the fold-change of expression.

Immunohistochemical staining. All tissues were paraffin-embedded and obtained from the Department of Pathology at the First Affiliated Hospital of Nanjing Medical University. Paraffin-embedded tissues were cut into 4- μ m sections, and incubated with the rabbit anti-human IGF-1R polyclonal antibody (cat. no. ab39398; Abcam, Cambridge, MA, USA; dilution, 1:100) overnight at 4°C. SP-9000 Histostain™-Plus kits (ZSGB-BIO, Beijing, China) were used according to the manufacturer's instructions. Scoring was measured according to the cell cytoplasm staining pattern: 0, no cytoplasmic staining; 1, weak cytoplasmic staining; 2, moderate cytoplasmic staining; and 3, strong cytoplasmic staining.

Cell transfection. The miR-497 mimic, miR-497 inhibitor, miRNA mimic negative control (NC) (a miRNA mimic) and miRNA inhibitor NC were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China) and were transfected using Lipofectamine® 2000 transfection reagent (Invitrogen™; Thermo Fisher Scientific) according to the manufacturer's instructions. The sequences were as follows: miR-497 mimic, 5'-CAGCAGCACACUGUGGUUUGU-3'; NC, 5'-UUCUCCGAACGUGUCACGUTT-3'; miR-497 inhibitor, 5'-ACAAACACAGUGUCUGCUG-3'; and miRNA inhibitor NC, 5'-CAGUACUUUUGUGUAGUACAA-3'.

Cell proliferation assays. Cells were seeded at a density of 2,000-5,000 cells/well in 96-well plates in 100 μ l complete

media. Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was used to measure cell viability according to the manufacturer's instructions. Briefly, cells were seeded at a density of 2,000-5,000 cells/well in 96-well plates in 100 μ l DMEM containing 10% FBS (HyClone™; GE Healthcare Life Sciences), penicillin (50 U/l; Invitrogen™; Thermo Fisher Scientific, Inc.) and streptomycin (50 μ g/ml; Invitrogen™; Thermo Fisher Scientific, Inc.) and cultured for 6 days in 5% CO₂ in a 37°C humidified incubator. Next, 10 μ l CCK-8 solution was added to each well and incubated at 37°C for 1 h. The absorbance was then calculated at a wavelength of 450 nm using a microplate reader (ELX808; BioTek Instruments, Inc., Winooski, VT, USA). Each experiment was repeated at least three times.

Colony formation assays. To examine the effect of upregulated or downregulated miR-497 expression on the proliferation of HCC cell lines, cells transfected with miR-497 mimics, miR-497 inhibitor, NC and miRNA inhibitor NC were used for colony formation assays. Each cell type was seeded into 6-well plates (500 cells/well) and cultured for 3 weeks. Cultures were stained with 0.4% crystal violet (Beyotime Biotech, Jiangsu, China). Colonies of >2 mm were counted and the mean number of colonies per well was calculated from three wells for each experiment. Each experiment was repeated at least three times.

Soft agar colony formation assays. Cells were transfected with miR-497 mimics, miR-497 inhibitor, NC and miRNA inhibitor NC, suspended in 0.5 ml of 1% low melting point agarose with complete culture media, and layered on top of 0.5 ml of 2% low melting point agarose (Department of Liver Transplantation Center, The First Affiliated Hospital of Nanjing Medical University) in 24-well plates. Cell counts varied from 2,000 to 5,000 cells depending on the cell line. Plates were incubated for 2 weeks in a 37°C humidified incubator with 5% CO₂. Colonies in at least 6 random microscopic fields were counted (Nikon Eclipse 50i; Nikon Corporation, Tokyo, Japan) and photographed (AxioCam MRc5; Carl Zeiss Shanghai Co., Ltd., Shanghai, China). All experiments were repeated three times.

Tumorigenicity assays in nude mice. Male BALB/c nude mice (aged 3-4 weeks) were purchased from the Department of Laboratory Animal Center of Nanjing Medical University. Cells with differential miR-497 expression were injected subcutaneously into the lateral root of the anterior limb of the nude mice (5.00×10^6 cells/mouse; 6 mice/experimental group). Tumor size was measured every third day following injection. At 3 weeks after injection, mice were sacrificed via cervical dislocation and tumors were excised and photographed. The weights of the tumors were also recorded. Experimental animals were maintained in accordance with Institutional Animal Care and Use Committee guidelines (38).

Western blot analyses. Cell lysates were prepared using cold lysis buffer containing 25 mM Tris-Cl (pH 7.5), 5 mM EDTA, 1% sodium dodecyl sulfate (SDS), and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA).

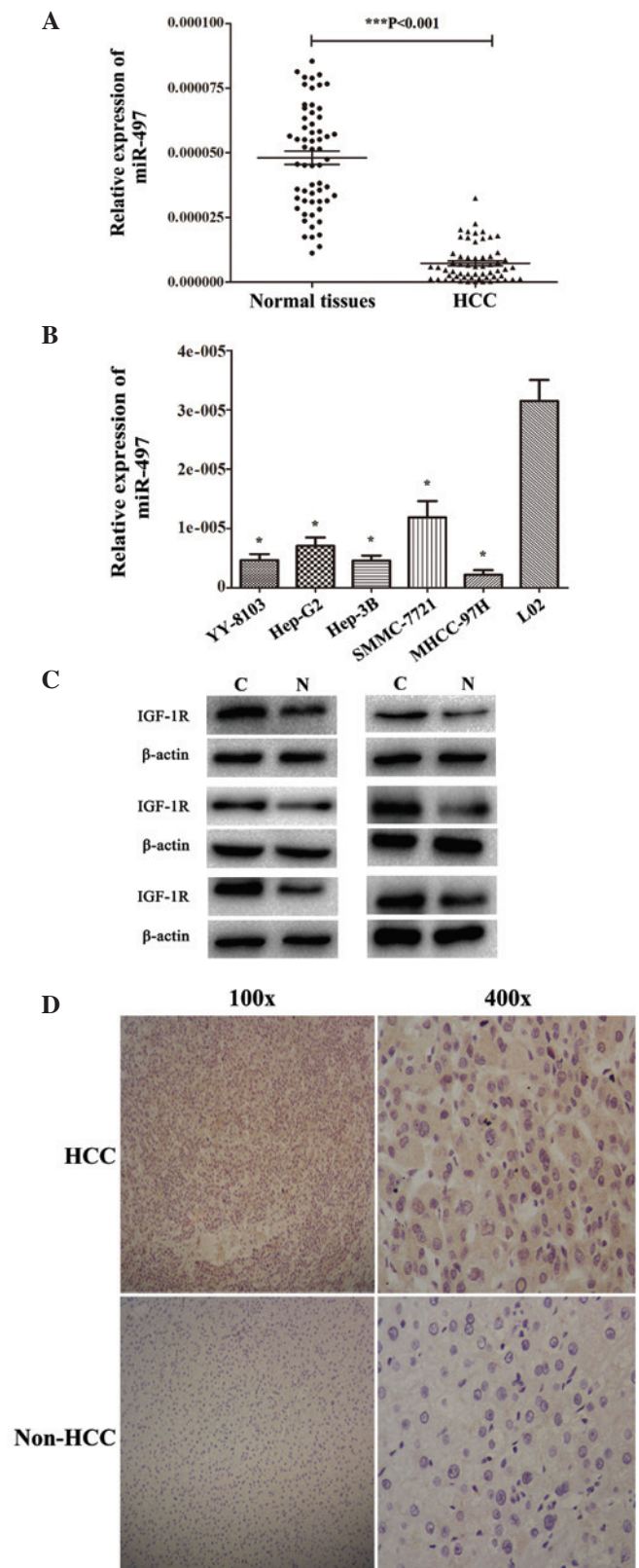


Figure 1. Downregulation of miR-497 and upregulation of IGF-1R in HCC. qPCR results indicated that miR-497 expression was downregulated (A) in 60 paired HCC specimens relative to matched adjacent normal tissue ($***P < 0.001$), and (B) in five HCC-derived cell lines compared with L02 normal human liver cells ($P < 0.05$). (C) Representative western blot results showing IGF-1R protein upregulation in HCC specimens. (D) Representative immunohistochemistry results showing upregulation of IGF-1R in HCC compared with non-HCC specimens (magnification, x100 and x400). miR, microRNA; IGF-1R, insulin-like growth factor-1 receptor; HCC, hepatocellular carcinoma; qPCR, quantitative polymerase chain reaction; C, cancer tissue; N, adjacent non-cancerous tissue.

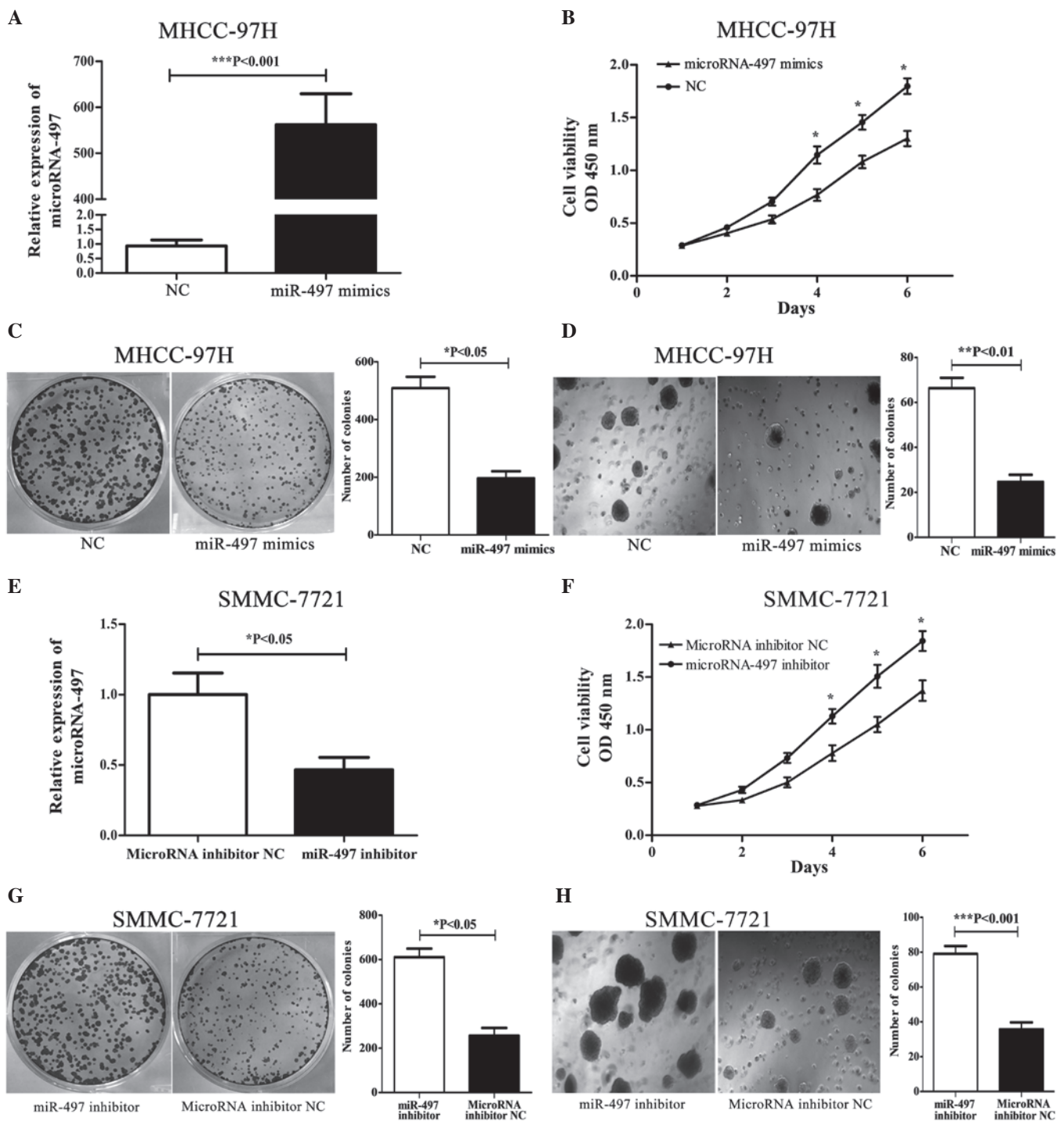


Figure 2. Differential expression of miR-497 influences cell proliferation and colony formation. (A) qPCR confirmed that MHCC-97H cells transfected with miR-497 mimics exhibit increased miR-497 expression compared with NC-transfected cells (***P<0.001). (B) miR-497 overexpression inhibits MHCC-97H cell proliferation (P<0.05). (C) miR-497 overexpression inhibits colony formation of MHCC-97H cells (P<0.05). (D) Overexpression of miR-497 inhibits soft agar colony formation of MHCC-97H cells (**P<0.01). (E) SMMC-7721 cells transfected with the miR-497 inhibitor had decreased miR-497 expression compared with inhibitor NC-transfected cells (P<0.05). (F) miR-497 silencing promotes proliferation of SMMC-7721 cells (P<0.05). (G) miR-497 silencing promotes colony formation of SMMC-7721 cells (P<0.05). (H) miR-497 silencing promotes soft agar colony formation of SMMC-7721 cells (***P<0.001). miR, microRNA; NC, negative control; OD, optical density.

Protein concentration was subsequently determined using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Rockford, IL, USA). After boiling for 5 min, samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked for 1 h at room temperature with 5% blocking

buffer (Inner Mongolia Yili Industrial Group Co., Ltd., Inner Mongolia, China), washed three times with Tris-buffered saline containing 0.1% Tween-20, and incubated overnight at 4°C with rabbit anti-human polyclonal primary antibodies against IGF-1R (cat. no. ab39398; dilution, 1:100; Abcam), p21 (cat. no. ab109199; dilution, 1:1,000; Abcam), p27 (cat. no. ab32034; dilution, 1:1,000; Abcam),

phospho- (p-) Ser473 Akt (cat. no. ab126433; dilution, 1:1,000; Abcam), p-glycogen synthase kinase 3 β (GSK3 β ; cat. no. ab75745; dilution, 1:1,000; Abcam) and β -actin (cat. no. ab119716; dilution, 1:1,000; Abcam). After the membranes were washed, they were incubated for 1 h at room temperature with the mouse anti-rabbit IgG secondary antibody (cat. no. bs-0295M; dilution, 1:1,000; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China). Proteins were detected by enhanced chemiluminescence (ECL) using a Pierce™ ECL Western Blotting detection system (Thermo Fisher Scientific, Inc., Rockford, IL, USA). β -actin was used as the internal control.

Statistical analyses. Statistical analyses were performed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Quantitative data are presented as the mean \pm standard deviation. Differences between two groups were assessed using a Student's t-test (two-tailed). $P < 0.05$ was considered to indicate statistically significant differences.

Results

HCC exhibits decreased miR-497 expression and increased IGF-1R expression. qPCR was performed to measure miR-497 expression levels in HCC and adjacent non-cancerous liver tissues from 60 patients. miR-497 expression was observed to be decreased compared with matched normal liver tissues in 42/60 (70.0%) HCC specimens ($P < 0.001$; Fig. 1A). Next, the expression of miR-497 was evaluated in five HCC-derived cell lines using qPCR. miR-497 expression was significantly reduced in all HCC-derived cell lines (YY-8103, HepG2, Hep3B, SMMC-7721 and MHCC-97H) compared with L02 normal human liver cells (Fig. 1B). Of the five HCC-derived cell lines, MHCC-97H cells exhibited the lowest level of miR-497 expression, whereas SMMC-7721 exhibited the highest level of miR-497 expression (Fig. 1B). One of the predicted common targets of miR-497 is IGF-1R (http://mirtarbase.mbc.nctu.edu.tw/php/search.php?q=search_exact&searchword=hsa-miR-497-5p). Therefore, western blot analysis was conducted to measure IGF-1R protein levels in HCC and adjacent non-cancer tissue. The results indicated that IGF-1R protein expression was increased in HCC specimens compared with matched normal liver tissues (Fig. 1C). Immunohistochemistry was also used to evaluate IGF-1R protein expression in HCC specimens and paired normal tissues in the same 60 matched samples. Of these specimens, 24/60 (40.0%) cancerous specimens exhibited no or weak positive staining, whereas 45/60 (75.0%) non-HCC tissues showed no or weak positive staining (Fig. 1D). Collectively, these data indicate that miR-497 expression is decreased and IGF-1R expression is increased in HCC tissues.

miR-497 overexpression inhibits proliferation and colony formation in MHCC-97H cells. To overexpress miR-497, miR-497 mimics were transfected into MHCC-97H cells, which exhibited the lowest level of miR-497 expression among the 5 HCC-derived cell lines. qPCR was used to evaluate miR-497 expression in transfected cells at 24 h posttransfection. In miR-497 mimic-transfected cells, miR-497 expression was significantly

higher compared with that of miR-497 NC-transfected cells (Fig. 2A). To investigate the effects on proliferation in miR-497 mimic-transfected cells, cell growth was monitored for 6 days. miR-497 mimic-transfected MHCC-97H cells exhibited significantly reduced cell proliferation compared with that of miR-497 NC-transfected cells (Fig. 2B) ($P < 0.05$). MHCC-97H cells with upregulated miR-497 expression were subjected to colony formation assays. As shown in Fig. 2C, miR-497 overexpression in MHCC-97H cells significantly inhibited colony formation relative to MHCC-97H cells transfected with miR-497 NC ($P < 0.05$); furthermore, the majority of clones were smaller than those of control cells. Next, soft agar assays were utilized to assess colony formation; these are the most stringent assays for detecting the proliferative ability of cells (39). Reduced colony formation was observed in soft agar (Fig. 2D) that had been seeded with MHCC-97H cells transfected with miR-497 mimics, compared with that seeded with miR-497 NC-transfected cells ($P < 0.01$). These results indicate that miR-497 inhibits tumor cell growth *in vitro*.

miR-497 knockdown promotes proliferation and colony formation in SMMC-7721 cells. To knockdown miR-497, a miR-497 inhibitor was transfected into SMMC-7721 cells, which exhibited the highest level of miR-497 expression among the 5 HCC-derived cell lines. qPCR was performed to assess the efficiency of miR-497 knockdown in these cells, confirming that miR-497 expression in miR-497 inhibitor-transfected cells was significantly lower compared to miRNA inhibitor NC-transfected cells (Fig. 2E). miR-497 inhibitor-transfected SMMC-7721 cells (Fig. 2F) exhibited enhanced cell proliferation compared with miRNA inhibitor NC-transfected cells ($P < 0.05$). Next, SMMC-7721 cells with downregulated miR-497 expression were subjected to colony formation assays. As shown in Fig. 2G, decreased miR-497 expression in SMMC-7721 cells significantly promoted colony formation relative to cells transfected with the miRNA inhibitor NC ($P < 0.05$). Enhanced colony formation in soft agar (Fig. 2H) was also observed in SMMC-7721 cells transfected with the miR-497 inhibitor compared with miRNA inhibitor NC-transfected cells ($P < 0.001$).

Differential expression of miR-497 affects tumorigenesis in nude mice. The effects of differential miR-497 expression on the tumorigenic potential of HCC cells were investigated *in vivo*. MHCC-97H cells with upregulated miR-497 expression and SMMC-7721 cells with downregulated miR-497 expression were injected subcutaneously into BALB/c nude mice. Tumor size was measured on every third day following injection. After 3 weeks, mice were sacrificed and the excised tumors were photographed and weighed. Results are shown in Fig. 3. Compared to mice injected with MHCC-97H cells transfected with miR-497 NC, mice injected with MHCC-97H cells overexpressing miR-497 exhibited smaller tumors during the same time period, and the mean tumor volumes and weights were significantly lower than the control group ($P < 0.05$) (Fig. 3A, C, and E). Compared with mice injected with miRNA inhibitor NC-transfected SMMC-7721 cells, mice injected with miR-497-underexpressing SMMC-7721 cells exhibited an increased capacity for tumorigenesis ($P < 0.05$) (Fig. 3B, D and F). Taken together, these

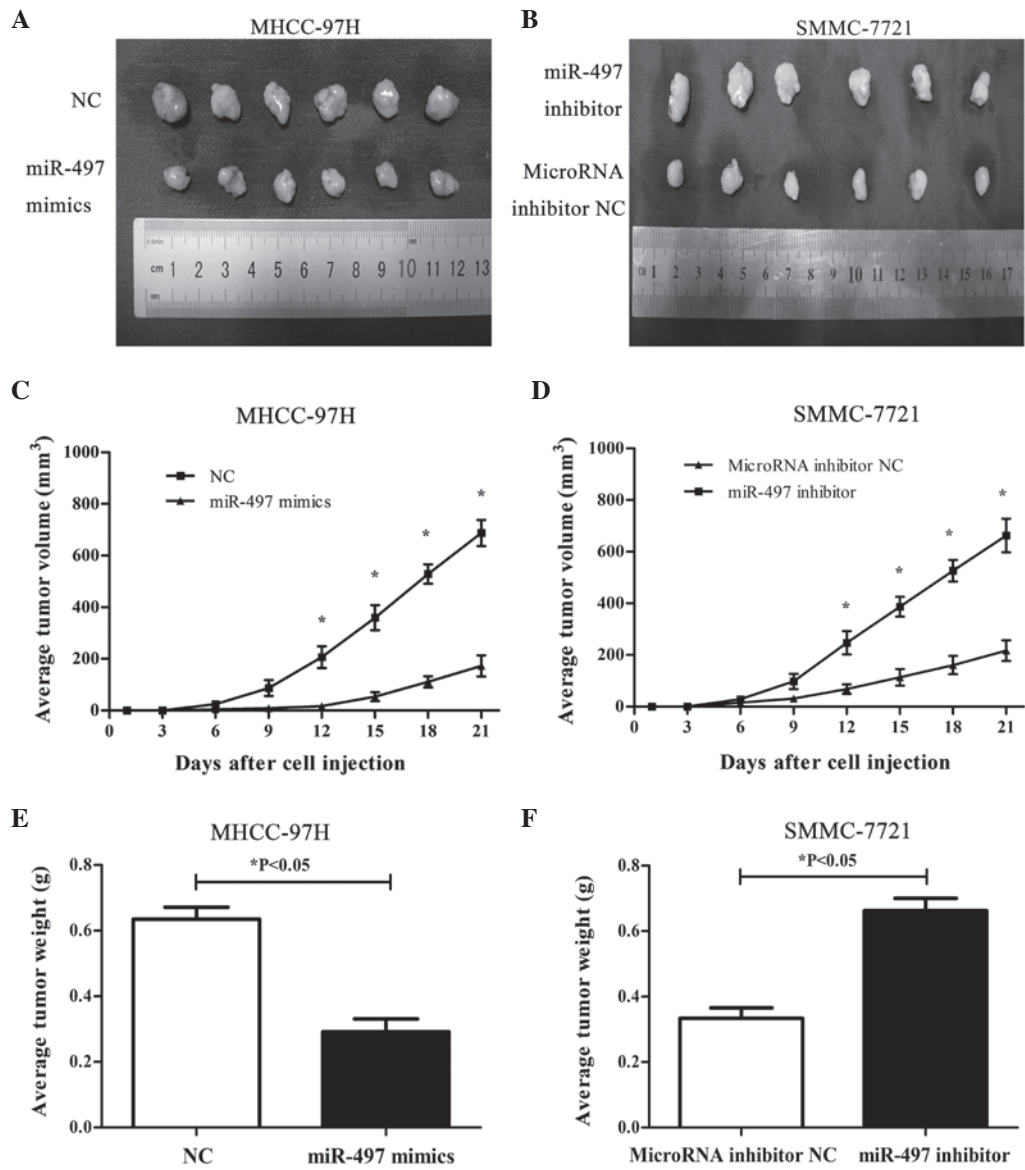


Figure 3. The effect of miR-497 expression on tumorigenesis in nude mice. (A and B) Tumors were excised 21 days after injection of cells. (C and D) Tumor volumes were measured every third day after injection (**P*<0.05). (E and F) The average (mean) weight of tumors in each group was assessed (**P*<0.05). miR, microRNA; NC, negative control.

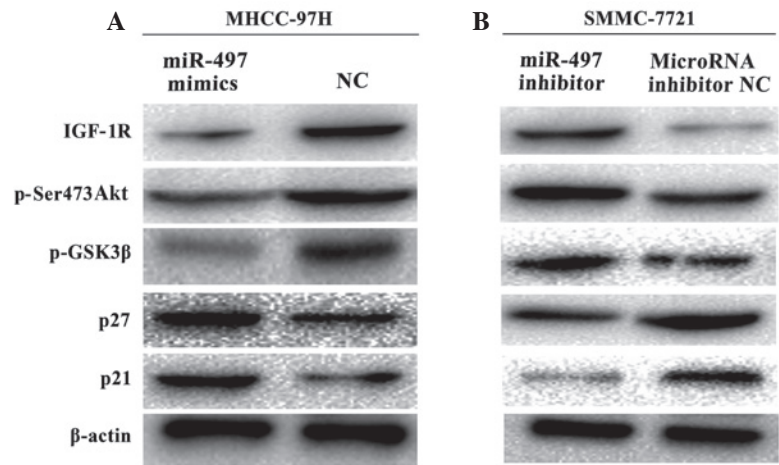


Figure 4. Western blot analyses of the phosphoinositide 3-kinase/Akt signaling pathway and cyclin pathway-related proteins. (A) Upregulation of p21 and p27 and downregulation of IGF-1R, pSer-473 Akt and p-GSK3β in miR-497-overexpressed MHCC-97H cells. (B) Downregulation of p21 and p27, and upregulation of IGF-1R, pSe-473 Akt and p-GSK3β in miR-497-silenced SMMC-7721 cells. miR, microRNA; NC, negative control; IGF-1R, insulin-like growth factor-1 receptor; GSK3β, p-glycogen synthase kinase 3 β.

results strongly suggest that miR-497 inhibits tumor cell growth and tumorigenicity *in vivo*.

miR-497 expression affects PI3K/Akt signaling and the expression of cyclin pathway-related proteins. To determine the mechanism(s) by which miR-497 regulates tumor growth and progression, potential miR-497-regulated molecules were examined in HCC cell lines with upregulated or downregulated miR-497 expression. Protein expression data revealed upregulation of p21 and p27, and downregulation of IGF-1R, p-Ser473 Akt and p-GSK3 β in miR-497-overexpressing MHCC-97H cells (Fig. 4A). Conversely, miR-497 silencing by the miR-497 inhibitor in SMMC-7721 cells led to downregulation of p21 and p27, and upregulation of IGF-1R, p-Ser-473 Akt and p-GSK3 β (Fig. 4B).

Discussion

HCC is the fifth most frequent cancer worldwide (40). A series of risk factors contribute to HCC occurrence, including infection with hepatitis B and C viruses, cirrhosis, chemical exposure, radiation and type 2 diabetes (41,42). Because surgical resection is only suitable for patients diagnosed with early stage disease, systemic chemotherapy remains an indispensable treatment option despite serious adverse reactions (43). Recently, increasing evidence suggests that miRNAs are involved in hepatocarcinogenesis, thus opening new avenues of investigation into the underlying molecular mechanism(s) of HCC, as well as providing potential new therapeutic targets (44).

The current study focused on miRNA-497, which was previously demonstrated to exhibit decreased expression in numerous tumor types, and which may function as a tumor suppressor (12-20). The results confirmed that the expression levels of miRNA-497 are decreased in HCC tumor tissues or HCC-derived cell lines compared with adjacent non-cancerous tissues or normal human L02 hepatocytes. Previously, Furuta *et al* (21) reported that miR-497 suppressed cell growth by targeting multiple cell-cycle regulators in HCC (21). These results were consistent with those of the current *in vitro* experiments in which exogenous overexpression of miRNA-497 was observed to inhibit MHCC-97H colony formation and tumor growth.

IGF-1R, a target gene of miR-497, was also selected for investigation; overexpression of this gene has been reported in human cervical cancer and colorectal cancer (12,20). Furthermore, several online databases, including miRanda (<http://www.microrna.org/microrna/getMrna.do?gene=3480&utr=31402&organism=9606&matureName=hsa-miR-497>) and miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/search.php?q=search_exact&searchword=hsa-miR-497-5p), indicated that IGF-1R mRNA contains miR-497 binding sites. IGF-1R belongs to the receptor tyrosine kinase family, and is activated by insulin-like growth factor (IGF)-1 and IGF-2 with high affinities. IGF-1R is a crucial component of the IGF axis that promotes cell proliferation, migration, and transformation (45,46). Previous studies have reported that IGF-1R expression is increased in HCC and is closely associated with tumor progression (34,35). In the current study, western blot and immunohistochemical analyses

revealed that IGF-1R was upregulated in HCC samples. As the PI3K/Akt signaling pathway is often activated in HCC, and is partially activated by IGF-1R (29,30,47), western blot analyses were employed to investigate components of this pathway. The results revealed that miR-497 overexpression reduced IGF-1R expression and decreased PI3K/Akt pathway activation. Changes were detected in downstream targets, including decreased phosphorylation of Akt (Ser473) and GSK3 β , and increased expression of p27 and p21. Conversely, miR-497 silencing resulted in increased IGF-1R expression and elevated activation of PI3K/Akt signalling. These results indicate that miR-497 regulates PI3K/Akt pathway activation, consistently with results from a previous study on human colorectal cancer (12). Notably, both miRNA-497 and p53 cluster at 17p13.1 (21), and wild-type p53 inhibits IGF-1R expression, whereas mutant p53 increases IGF-1R expression (48,49). These findings suggest that abnormal transcription or translation of the chromosome 17p13.1 fragment may play a crucial role in hepatocarcinogenesis.

In conclusion, the current results verify that miRNA-497 downregulation occurs frequently during hepatocarcinogenesis. miRNA-497 overexpression may suppress cellular growth by targeting IGF-1R and inhibiting activation of the PI3K/Akt pathway in HCC-derived cell lines. Currently, a series of antibodies and small molecule inhibitors targeting the IGF axis may provide an alternative strategy in the management of HCC (50). Therefore, artificial upregulation of miRNA-497 may also provide a new therapeutic option for HCC.

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