MicroRNA-497 inhibits tumor growth through targeting insulin receptor substrate 1 in colorectal cancer

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Abstract. MicroRNAs (miRNAs) have been demonstrated to serve an important role in diverse biological processes and cancer progression. Downregulation of microRNA-497 (miR-497) has been observed in human colorectal cancer (CRC) tissues, but the function of miR-497 in CRC has not been well investigated. In the present study, it was demonstrated that expression of miR-497 was significantly downregulated in human CRC tissues compared to adjacent normal tissues. Enforced expression of miR-497 inhibited proliferation, migration and invasion abilities of CRC cell lines SW1116 and SW480. Furthermore, overexpression of miR-497 inhibited phosphoinositide 3-kinase/AKT and mitogen-activated protein kinase/extracellular signal-regulated kinase signaling by targeting insulin receptor substrate 1 (IRS1). In human clinical specimens, IRS1 was inversely correlated with miR-497 in CRC tissues. Collectively, the results of the present study demonstrate that miR-497 is a tumor suppressor miRNA and indicate its potential application for the treatment of human CRC in the future.

Introduction

Colorectal cancer (CRC) is the fourth most commonly diagnosed cancer and the third leading cause of cancer-associated mortality in men and women (1). A number of patients are diagnosed in the advanced disease stage, despite efforts and improvements in early diagnosis (2). Although a variety of therapeutic options are available for CRC patients, including surgery, chemotherapy and radiotherapy, the five-year survival rate of CRC has not significantly improved (3). Previous studies have demonstrated that genetic and epigenetic alterations are involved in the tumorigenesis of CRC, including the activation of oncogenes and/or the suppression of tumor suppressor genes. Increasing evidence suggests that microRNAs (miRNAs/miRs) may serve key roles in the development of CRC (4-6).

miRNAs, a class of endogenous single-stranded non-coding RNAs, have been associated with various types of cancer (7). miRNAs serve an essential role in the regulation of gene expression and are involved in numerous important physiological and pathological processes, including development, differentiation and tumorigenesis (8-10). By binding the 3'-untranslated region (3'-UTR) of mRNA, miRNA suppresses protein synthesis through mRNA degradation or translational repression. As a result, miRNAs may act as either tumor suppressors or oncogenes (11-13). It is becoming increasingly evident that miRNAs serve important roles in cancer etiology. As a tumor suppressor gene in several cancer types, miR-497 is able to affect tumor cell growth, migration, invasion and apoptosis (14,15). To date, certain genes have been identified as miR-497 targets, including Ndp1, Cyclin E1, B-cell lymphoma 2 and insulin-like growth factor 1 receptor (IGF1R) (14,16-18). However, the role and underlying mechanism of miR-497 in regulating tumorigenesis remains to be further elucidated. Notably, it has been reported that miR-497 regulates malignant behavior of CRC cells by targeting IGF1R (16). In the present study, it was observed that miR-497 targeted insulin receptor substrate 1 (IRS1), which is characterized as a typical cytosolic adaptor protein in both insulin receptor (IR) and IGF1R signaling. Studies have indicated that nuclear IRS1 is able to participate in modulating the transcriptional activity of genes involved in cell growth and proliferation (19,20). Nuclear IRS1 binds β-catenin and works as a transcriptional modulator to stimulate cyclin D1 and c-myc promoter activities in a number of cancer types, where it acts as an oncogene, including in pancreatic (21) and breast cancer (22). Epidemiological investigation has revealed that IRS1 is important in the etiology of CRC (23).
It has been reported that miR-497 may serve roles in CRC via affecting various signaling pathways (16). In the present study, the aim was to identify the roles of miR-497 and its molecular and cellular mechanisms in CRC. Ectopic expression of miR-497 inhibited proliferation, migration and invasion of CRC cells by suppressing a key target, IRS1. Furthermore, the present study defined the molecular mechanism of the tumor suppressive function of miR-497 by inhibiting both phosphoinositide 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathways via IRS1 suppression. The results of the present study revealed that miR-497 expression was significantly downregulated in human CRC tissues compared with adjacent paired normal controls. IRS1 expression in CRC tumors was negatively correlated with miR-497 expression. The results of the present study revealed a novel mechanism of miR-497 in CRC, and demonstrate its potential to be used as a novel strategy to develop miR-497-based therapeutics.

Materials and methods

Cell culture and clinical tissues. Human CRC cell lines SW1116 and SW480 (purchased from Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and HEK-293T cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 mg/ml streptomycin. All cells were incubated at 37˚C in an atmosphere of 5% CO₂.

Colon cancer tissues and adjacent normal tissues were collected from clinical patients undergoing colon cancer resection. All tissue samples were immediately snap-frozen in liquid nitrogen following surgery. All human CRC samples were divided into Grade I, Grade II and Grade III-IV according to the WHO classification (24). In total, 50 pairs of CRC tissues and adjacent normal tissues from patients who underwent surgical operations at The Third Affiliated Hospital of Soochow University (Changzhou, China) from August 1, 2013 to July 31, 2014, were obtained for the study. Written informed consent was obtained from all patients. The present study was approved by the review board and ethics committee of The Third Affiliated Hospital of Soochow University.

Lentiviral packaging and stable cell line establishment. The Lentiviral Packaging kit was used (Thermo Fisher Scientific, Inc.) for stably overexpressing miR-497 in CRC cells. Lentivirus carrying miR-497 or negative control (miR-NC) was packaged following according to the manufacturer's protocol. Lentivirus was packaged in HEK-293T cells and secreted into the medium. Cells were transfected with lentivirus carrying miR-497 or miR-NC in the presence of polybrene (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and selected by puromycin (Sigma-Aldrich; Merck KGaA) for 2 weeks to obtain stable cell lines.

miRNA mimic transfection. Cells were seeded into 6, 12, 24, or 96-well plates and incubated at 37˚C and 5% CO₂ overnight.

miR-497 mimics and miR-NC were chemically synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells at 50-70% confluence were transfected with miR-497 or miR-NC using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Transfected cells were harvested at 24 or 48 h following transfection.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cultured cells using 1.0 ml of Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and purified RNA was stored at -80˚C prior to further analysis. RT-qPCR analysis for mature miR-497 was performed in triplicate using the PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. Briefly, 500 ng total RNA was reverse transcribed into cDNA, and qPCR was performed using SYBR Premix DimerEraser (Takara Biotechnology Co., Ltd.) on a 7900HT system. The thermocycling conditions were as follows: Pre-denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec, 55˚C for 30 sec and 72˚C for 31 sec. The sequences of the primers used for RT-qPCR were as follows: miR-497 RT, 5'-CTCAACTTGTGTCGGAGA GTCGGCAATTCAGTTGAGAA-3'; miR-497-forward (F), 5'-ACACTCCAGTGGCCGACACACTGGG-3'; miR-497-reverse (R), 5'-TGTTGTCGTCGGAGTCG-3'; U6 RT, 5'-AACCGTTCACGAATTTGCCGA-3'; U6-F, 5'-CTCG TCTCGGGCAGAC-3'; and U6-R, 5'-TGGTGTCGTCGGAGTCG-3'. The miR-497 expression in each group was determined relative to that of U6, and fold changes were calculated by relative quantification (2 ΔΔCq) (25).

Cell proliferation assay. Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) assay was used to determine cell viability. Cells were seeded at a density of 2,000 cells per well in 96-well plates and cultured as described above for 48 h following transfection. After 24, 48, 72 and 96 h incubation, CCK-8 was added into each well, followed by an additional 2 h incubation at 37˚C. Absorbance at a wavelength of 450 nm was subsequently determined. Experiments were performed in triplicate.

Wound healing assay. Cells were transfected with miR-497 or miR-NC according to the manufacturer's protocol, and subsequently cultured to 95% confluence in 6-well plates. Cell monolayers were scratched using a 20 µl tip to form wound gaps and washed twice with PBS to remove the detached cells. After 24 h, the wound healing was photographed at various time points. The cell migration distances were measured in three different areas to indicate the migration ability of various cell treatments.

Invasion assay. The effect of miR-497 on tumor invasion was investigated using 24-well BD Matrigel invasion chambers (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. The transfected cells (5x10⁴) were seeded in the upper well of the invasion chamber containing serum-free RPMI-1640, and RPMI-1640 containing 10% FBS was applied to the lower chamber. After 24 h, any non-invading
cells on the top well were removed with a cotton swab, while cells in the bottom well were fixed with 3% paraformaldehyde and stained with 0.1% crystal violet. Images were captured in three independent fields (magnification, x10). The membrane was air-dried, soaked with 3% acetic acid (200 µl/well) at room temperature for 15 min and subsequently transferred to a 96-well plate. The absorbance at a wavelength of 570 nm was recorded (Synergy 2; BioTek Instruments, Inc., Winooski, VT, USA). Results were obtained from three independent experiments.

Western blotting. Cells were treated as previously described, and were harvested after 24 h and lysed in radioimmunoprecipitation assay buffer supplemented with protease inhibitors (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 1% deoxycholate acid, 0.1% SDS, 2 mM DTT, 1 mM sodium orthovanadate, 2 mM leupeptin and 2 mM pepstatin) on ice for 30 min (26). Following centrifugation, protein concentrations were determined by the bicinchoninic acid method (Beyotime Institute of Biotechnology, Haimen, China), and 20 µg protein was then separated by 10% SDS-PAGE. Subsequently, protein was electrically transferred onto a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membrane was incubated with anti-IRS1 (1:1,000; catalog no. CST 2382; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-GAPDH (1:5,000; catalog no. MB001; Bioworld Technology, Inc., St. Louis Park, MN, USA) antibodies at 4°C overnight, followed by incubation at room temperature for 2 h with a secondary antibody (catalog no. 31460; Thermo Fisher Scientific, Inc.) diluted 1:2,000 for IRS1 detection and 1:5,000 for GAPDH detection. Antibodies against phosphorylated (p)-AKT (Ser473) (1:1,000; catalog no. CST 4060), AKT (1:2,000; catalog no. CST 9272), p-ERK1/2 (1:1,000; catalog no. CST 14474) and ERK1/2 (1:2,000; catalog no. CST 4348) were purchased from Cell Signaling Technology, Inc., and were incubated at 4°C overnight, followed by incubation at room temperature for 2 h with the aforementioned secondary antibody a 1:2,000 dilution. ECL Detection System (Thermo Fisher Scientific, Inc.) was used for protein signal detection. The density of the signals was quantified using ImageJ software with the ChemiDoc Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). GAPDH was used as a control for normalization.

Luciferase reporter assay. Prediction of miR-497 binding sites was performed using TargetScan software using the key words ‘IRS1’ and ‘human species’. TargetScan (www.targetscan.org) and miRanda (www.microrna.org) predict biological targets of miRNAs by searching for the presence of conserved 8mer, 7mer and 6mer sites that match the seed region of each miRNA (27). A fragment of 3'-UTR of IRS1 containing the putative miR-497 binding site was amplified by PCR. To generate a construct containing the mutant miR-497 binding site, two nucleotides corresponding to the 5'-seeding region of the miR-497 binding site on the wild type fragment were substituted. Its complementary sequence in the 3'-UTR of IRS1 (UGCUGCU) was replaced by UCCACCA. The PCR products were digested using SacI and HindIII, inserted into pMIR-REPORTER (Promega Corporation, Madison, WI, USA) and validated by DNA sequencing. Constructs were transfected into HEK-293 cells in 24-well plates and co-transfected with miR-497 or miR-NC. Luciferase assays were performed 24 h post-transfection using the Dual Luciferase Reporter Assay system (Promega Corporation).

Xenograft studies. For tumor growth assay, male nude mice [BALB/cA-nu (nu/nu), 6-week-old, weighting 20-25 g] were purchased from Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China), and animals were maintained under special pathogen-free (SPF) conditions. Mice were housed at a temperature of 22-25°C, relative humidity of 40-60% and 10 h light/14 h dark cycle, with access to food and water ad libitum. Animal protocols were approved by the Animal Welfare Committee of Soochow University (The Third Affiliated Hospital of Soochow University). Aliquots of cells (5x10^4) were suspended in 150 µl of FBS-free RPMI-1640 medium and subcutaneously injected into each side of the posterior flank of nude mice. Tumor size was measured using vernier calipers every 2 days when they became visible, and the tumor volume was calculated according to the formula: Volume = 0.5 x length x width^2. Mice were sacrificed on day 22 following injection of tumor cells, and xenografts were collected. The animals were euthanized by cervical dislocation (28).

Statistical analysis. All experiments were performed in triplicate, and data were analyzed with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). The correlation between the expression of miR-497 and IRS1 in CRC tissues was analyzed using Spearman's rank test. Statistical evaluation for data analysis was determined by the Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-497 is significantly downregulated in CRC tissue. To identify the role of miR-497 in the development of colorectal cancer, the expression level of miR-497 was analyzed in 50 pairs of CRC tissues and adjacent normal tissues. RT-qPCR analysis revealed that the miR-497 level was significantly downregulated in CRC tissues (Fig. 1A). It was also observed that reduced levels of miR-497 in CRC patients were positively correlated with the status of pathology classification (Fig. 1B). These results indicated that the progressive loss of miR-497 may be associated with CRC disease progression.

miR-497 suppresses CRC cell growth, migration and invasion in vitro. To investigate the direct role of miR-497 in CRC cells, the present study established stable cell lines by transfecting SW1116 and SW480 cells with miR-497 overexpressing lentiviral vector (Lv-miR-497) or a control lentiviral empty vector (Lv-miR-NC), followed by puromycin selection. Subsequently, CRC cell proliferation was detected in vitro. Cell viability assay indicated that overexpression of miR-497 significantly reduced the cell proliferation rate at 48 h following cell seeding in SW1116 and SW480 cells,
compared with the LV-miR-NC group (Fig. 2A). Since invasion and migration are key characteristics of malignant tumors, the present study investigated the effects of miR-497 on invasion and migration in vitro. Forced expression of miR-497 also markedly suppressed the invasion of SW1116 and SW480 cells in migration assays, as well as wound healing assays (Fig. 2B and C).

\textit{miR-497 inhibits AKT and ERK1/2 signaling pathways via targeting IRS1.} To investigate the underlying mechanism of miR-497 in CRC, the present study analyzed TargetScan and miRanda databases. It was observed that miR-497 likely regulates the IRS1 gene, since the 3'-UTR of IRS1 contained the binding site for the seed region of miR-497. IRS1 is characterized as a typical cytosolic adaptor protein in both IR and IGF1R signaling. According to the putative binding site of miR-497 in the 3'UTR of the IRS1 gene, the present study initially constructed two types of plasmids containing the luciferase reporting gene and wild-type or mutant IRS1 3'UTR and cotransfected a miR-497 mimic into HEK-293T cells; cells co-transfected with a miR-497 mimic and wild-type IRS1 3'UTR demonstrated a significant decrease in luciferase activity. However, in the mutant group, no detectable change in luciferase activity was observed (Fig. 3A), suggesting that miR-497 suppressed the transcription of the IRS1 gene by targeting the putative 3'UTR of IRS1 mRNA independently. Western blotting analysis was conducted to determine IRS1 protein expression. The results revealed that the IRS1 expression in SW1116 and SW480 cells transfected with miR-497 mimics was downregulated at the protein level, compared with cells transfected with negative control (Fig. 3B). These data demonstrated that miR-497 directly targeted IRS1 by binding its seed region to the 3'-UTRs in CRC cells. The present study additionally examined the IRS1 expression at the protein level in human CRC specimens and normal tissues. The results demonstrated that the average expression level of IRS1 was significantly increased in tumor tissue compared with normal tissue (Fig. 3C). Further analysis revealed the significant reciprocal association of expression levels of IRS1 with miR-497 in the same human CRC tissue (\( r = -0.6247; \) Fig. 3D).

AKT and ERK1/2 signaling pathways act as major downstream regulators of IRS1 signaling, which are critical in mitogenesis and oncogenesis. Cellular levels of p-AKT and p-ERK1/2 were significantly changed in SW1116 and SW480 cells stably expressing miR-497 compared with miR-NC, but the changes in AKT and ERK1/2 were not statistically significant (Fig. 3E). To additionally investigate whether the overexpression of IRS1 affected the expression of p-AKT and p-ERK1/2, cells were co-transfected with or without pCMV6-IRS1 cDNA. The results of the present study demonstrated that forced expression of IRS1 restored miR-497-inhibited cellular levels of p-AKT and p-ERK1/2. These data revealed that miR-497 inhibited AKT and ERK1/2 signaling pathways via targeting IRS1 (Fig. 3E).

\textit{miR-497 inhibits tumor growth in vivo.} To investigate the effect of miR-497 on tumor growth, SW1116 cells overexpressing miR-497 or miR-NC were subcutaneously injected into the posterior flanks of nude mice (n=6). Xenograft tumor volumes were determined every 2 days after they had become visible. Nude mice were sacrificed on day 22 following injection of tumor cells, and xenografts were collected. Fig. 4A shows representative xenograft tumors. The average tumor weight of the miR-497 overexpression group was markedly reduced by 60% compared with that of the control (Fig. 4B). On day 16 post-implantation, the tumor growth of the miR-497 overexpression group was significantly reduced compared with that of the control group (Fig. 4C). Total proteins from representative tumor samples were analyzed by western blotting, and the results demonstrated that miR-497 suppressed the expression of IRS1 and p-AKT, as well as p-ERK1/2, \textit{in vivo} (Fig. 4D). Taken together, these results suggested that miR-497 inhibited tumor growth \textit{in vivo} via targeting IRS1 and other downstream signaling molecules.

\section*{Discussion}

Previous studies have demonstrated that miRNAs serve important roles in carcinogenesis by a number of mechanisms, and certain miRNAs have been reported to be correlated with clinical characteristics and outcomes (29,30). The role of...
Figure 2. Overexpression of miR-497 inhibits proliferation, invasion and migration in CRC cells. (A) Overexpression of miR-497 arrested cell proliferation in SW1116 and SW480 cells. (B) miR-497 overexpression reduced cell invasion in SW1116 and SW480 cells. (C) Cells were treated as shown. A sterile 20 µl pipette tip was used form a wound. The wound gaps were photographed and measured. Forced expression of miR-497 also markedly reduced the wound-healing rate. Data represent the mean ± standard deviation of three replicates. * Indicates significant difference at P<0.05; ** indicates significant difference at P<0.01. miR, microRNA; OD, optical density; NC, negative control.
Certain miRNAs in CRC has also been reported. For example, miR-378 is frequently downregulated in CRC and colorectal cell lines, and upregulation of miR-378 inhibits cell growth and enhances oxaliplatin-induced apoptosis in human CRC (31). miR-194 functions as a tumor suppressor in colorectal carcinogenesis via targeting phosphoinositide-dependent kinase-1/AKT2/X-linked inhibitor of apoptosis protein signaling pathway (32). Previous studies have demonstrated that miR-497 is downregulated in several cancer types, including CRC. Han et al (33) confirmed that miR-497 suppresses the proliferation of human cervical carcinoma HeLa cells by targeting cyclin E1. Another study demonstrated that miR-497 targeted insulin-like growth factor 1 receptor and inhibited proliferation and invasive behavior in colon cancer cells (16).
Consistent with previous studies, the present study identified that miR-497 was downregulated in CRC tissues compared with normal controls, and the degree of miR-497 suppression was negatively correlated with increased grades of human CRC malignancy. Notably, the present study further predicted IRS1 as a target of miR-497 by bioinformatic analysis. For the first time to the best of our knowledge, it was demonstrated that IRS1 was upregulated in CRC tissues and was inversely correlated with miR-497 levels. Thus, in combination with previous research, the present study demonstrated that miR-497 regulated the IGF1R/IRS1 signaling pathway, and may provide novel therapeutic strategies for CRC prevention and treatment.

IRS1 transmits signals from insulin or IGF receptors to activate PI3K/AKT and MAPK pathways, both of which are critical in mitogenesis and oncogenesis (34,35). It has been observed that the expression of IRS1 may promote proliferation in several cell lines (36-38). In the present study, the IRS1 oncogene was experimentally validated as a novel target of miR-497 in vitro and in vivo. Initially, luciferase reporter assay confirmed that miR-497 directly recognized the 3'-UTR of IRS1 transcripts. Furthermore, IRS1 expression was significantly abolished in CRC cells stably expressing miR-497. In addition, a negative correlation was observed between IRS1 protein and miR-497 in clinical samples. Finally, inhibition of IRS1 expression by miR-497 suppressed constitutive phosphorylation of AKT and ERK1/2. These results demonstrate that miR-497 is a tumor suppressor that inhibits the AKT and ERK1/2 signaling pathway through partly targeting IRS1.

In conclusion, the results of the present study provide the first evidence, to the best of our knowledge, that miR-497 serves a significant role in suppressing CRC cell growth via inhibition of IRS1. Although the present study confirmed that miR-497 was able to inhibit the phenotype of CRC by targeting IRS1, there may be other targets of miR-497, which could also affect the growth of CRC cells. However, the present study demonstrated that such an effect was exerted through the suppression of IRS1. Therefore, further studies are required to identify additional targets and signaling pathways of miR-497.

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