MicroRNA-497 inhibits cellular proliferation, migration and invasion of papillary thyroid cancer by directly targeting AKT3

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Abstract. Thyroid cancer is the most common tumor of the endocrine organs. Emerging studies have indicated the critical roles of microRNAs (miRs) in papillary thyroid cancer (PTC) formation and progression through function as tumor suppressors or oncogenes. The present study investigated the expression level and biological roles of miR-497 in PTC and its underlying mechanisms. It was demonstrated that the expression level of miR-497 was reduced in both PTC tissues and cell lines. Enforced expression of miR-497 suppressed PTC cell proliferation, migration and invasion. According to bioinformatics analysis, a luciferase reporter assay, reverse transcription-quantitative polymerase chain reaction and western blotting, RAC-γ serine/threonine-protein kinase (AKT3) was demonstrated to be the direct target gene of miR-497. In addition, AKT3 expression increased in PTC tissues and negatively correlated with miR-497 expression. Furthermore, downregulation of AKT3 also suppressed cell proliferation, migration and invasion of PTC, which had similar roles to miR-497 overexpression in PTC cells. Taken together, these results suggested that this newly identified miR-497/AKT3 signaling pathway may contribute to PTC occurrence and progression. These findings provide novel potential therapeutic targets for the therapy of PTC.

Introduction

Thyroid cancer is the most common tumor of the endocrine organ with 300,000 new cases and nearly 40,000 deaths each year worldwide (1). According to the histological characteristics, thyroid cancer can be divided into four categories, including papillary, follicular, medullary and anaplastic thyroid cancer (2). Papillary thyroid cancer (PTC), formed from follicular or parafollicular thyroid cells, is the most common thyroid type of cancer and makes up ~80% of all malignancies in thyroid (3,4). Currently, with the development of standard treatments, the vast majority of patients with PTC have an excellent prognosis (5); however, approximately 10-15% of patients present recurrence and metastasis (6). Furthermore, PTC patients diagnosed at advanced stage often suffer from surrounding structure metastasis, such as the throat, trachea, epiglottis, esophagus and cervical vessels (7). Given this, it is important to understand the mechanism underlying the progression of PTC and develop novel therapeutic strategies for the treatments of patients with this disease.

MicroRNAs (miRNAs/miRs) are a series of small, endogenous, non-coding and highly conserved RNA molecules of approximately 19-23 nucleotides (8). They mainly function as posttranscriptional regulators by directly binding to the 3'-untranslated regions (3’UTRs) of their target genes in a base-pairing manner, thus leading to mRNA cleavage or translation inhibition (9). It is well established that one single miRNA could negatively regulate a great deal of target genes as a result of their abundance and target specificity (10-12). For decades, miRNAs have been reported to serve key roles in multiple cellular processes, such as cell proliferation, apoptosis, cell cycle, development, differentiation, invasion, metastasis and tumorigenesis (13-15). Previously, miRNAs were demonstrated to be abnormally expressed in various types of human cancer, such as PTC (16), gastric cancer (17), glioma (18), bladder cancer (19) and colorectal cancer (20). Additionally, emerging studies have indicated the critical roles of miRNAs in tumor occurrence and progression through functioning as tumor suppressors or oncogenes (21-23). These findings suggested that miRNAs could be used as potential therapeutic targets for PTC therapy.

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The present study detected miR-497 expression in both PTC tissues and cell lines, and investigated its biological roles in PTC progression. The molecular mechanisms underlying the action of miR-497 in PTC were evaluated.

Materials and methods

Tissue specimens and cell lines. Primary PTC tissues and adjacent normal thyroid tissues were collected from 43 patients (age range, 35-67 years; median age, 48; 18 males and 25 females) with PTC who treated with surgery at The Seventh People's Hospital of Shanghai University of TCM (Shanghai, China). A total of 12 patients were diagnosed at stage I, 18 at stage II, 8 at stage III and 5 at stage IV. These patients did not receive neoadjuvant therapy. All tissues were snap-frozen immediately after surgery and stored at -80°C. The present study was approved by the Ethics Committee of The Seventh People's Hospital of Shanghai University of TCM, and written informed consent was also obtained from all patients.

TPC-1, K1, HTH83 and BCPAP human PTC cell lines and the HT-ori3 normal human thyroid cell line were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.), at 37°C in humidified 5% CO₂ cell incubator.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from homogenised tissues and cell lines was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The purity and concentration of total RNA was assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA was synthesised using a PrimeScript RT reagent kit (Takara, Otsu, Japan). qPCR was performed with SYBR Premix Ex Taq (Takara Bio, Inc.) on an Applied Biosystems 7900HT Real-Time PCR system (Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 30 sec and at 65°C for 45 sec. U6 small nuclear RNA (U6 snRNA) and GAPDH were used as internal controls for miR-497 and AKT3 mRNA expression, respectively. The primers used in the present study were as follows: miR-497, 5'-CCAGTTCGATTTTCTCAGTGTCGAGGTTGA-3' (forward) and 5'-GTGGCACTGGTCTCAGAGGTTTCA-3' (reverse); U6, 5'-GCTCCTCAGAGGTTTCA-3' (forward) and 5'-CGTGTCTCAGAGGTTTCA-3' (reverse); AKT3, 5'-AATGGGAACAGCTAGTCCCTCAGAC-3' (forward) and 5'-CTGTGCTCAGACAGCAGTCC-3' (reverse); and GAPDH, 5'-CCAGGTCGAGGCCAAGATTGTC-3' (forward) and 5'-CGTTGCTCTCTCAGAGGTTTCA-3' (reverse). Data were calculated using the 2-ΔΔCT method (24).

Transfection. Cells in FBS-free DMEM were seeded into six-well plates at a density of 60-70% confluence. After adherence, cells were transfected with miR-497 mimics, negative control miRNA mimics (miR-NC), AKT3 small interfering (si)RNA, NC siRNA, pCDNA3.1-AKT3 or pCDNA3.1 blank vector using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Inc.). After 6-8 h of incubation, culture medium was replaced by DMEM supplemented with 10% FBS. miR-497 mimics and miR-NC were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). AKT3 siRNA or NC siRNA were obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). pCDNA3.1-AKT3 and a pCDNA 3.1 blank vector were synthesized by the Chinese Academy of Sciences (Changchun, China).

Luciferase reporter assay. The wild-type (pGL3-AKT3-3'UTR Wt) and mutant (pGL3-AKT3-3'UTR Mut) AKT3 luciferase reporter vectors were synthesised by Shanghai GenePharma. HEK293T cells (ATCC) were seeded into 24-well plates at a density of 40-50% confluence. After incubation overnight, cells were transfected with pGL3-AKT3-3'UTR Wt or pGL3-AKT3-3'UTR Mut, together with miR-497 mimics or miR-NC, using Lipofectamine 2000. Transfected cells were cultured at 37°C in humidified 5% CO₂. At 48 h post-transfection, cells were collected and subjected to a luciferase reporter assay using Dual-Luciferase® Reporter Assay system (Promega Corporation, Madison, WI, USA). The relative luciferase activity was normalized with Renilla luciferase.
activity. Each assay was performed in triplicate and repeated three times.

**Western blot analysis.** Transfected cells were harvested and lysed in radioimmunoprecipitation assay buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA), blocked in 5% fat-free milk and Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature. Membranes were then incubated with mouse anti-human AKT3 (cat. no. sc-134254; 1:1,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or GAPDH (cat. no. sc-22323; 1:1,000 dilution; Santa Cruz Biotechnology, Inc.) monoclonal antibodies, at 4˚C overnight. Membranes were washed in TBST three times and probed with a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (cat. no. sc-2005; 1:5,000 dilution; Santa Cruz Biotechnology, Inc.) or GAPDH (cat. no. sc-32233; 1:1,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or GAPDH (cat. no. sc-32233; 1:1,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or GAPDH (cat. no. sc-32233; 1:1,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature. After three washes in TBST, the protein bands were visualized using an enhanced chemiluminescence detection reagent (Thermo Fisher Scientific, Inc.). Protein levels were determined by normalization to GAPDH. ImageJ software version 1.49 (National Institutes of Health, Bethesda, MD, USA) was used to semi-quantify blots by densitometry.

**Statistical analysis.** Data are expressed as the mean ± standard deviation and were analyzed using Student's t-test or one-way analysis of variance followed by Student-Newman-Keuls post hoc test using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Pearson correlation analysis was used to determine the correlation between miR-497 and AKT3 mRNA expression levels. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**miR-497 is low in PTC tissues and cell lines.** Firstly, miR-497 expression was analyzed in PTC tissues using RT-qPCR. As presented in Fig. 1A, miR-497 expression in PTC tissues was significantly downregulated compared with in adjacent normal thyroid tissues (P<0.05). Furthermore, analysis of miR-497 expression in four human PTC cell lines (TPC-1, K1, HTH83 and BCPAP) and the HT-ori3 normal human thyroid cell line indicated that expression levels of miR-497 were decreased in tumor cell lines as well (Fig. 1B, P<0.05). These results suggested that miR-497 may serve important roles in PTC progression.

**miR-497 inhibits cell proliferation, migration and invasion of PTC.** To examine the roles of miR-497 in PTC, TPC-1 and HTH83 cells were transfected with miR-497 mimics to increase its expression (Fig. 2A, P<0.05). MTT assay demonstrated that re-expression of miR-497 inhibited proliferation in TPC-1 and HTH83 cells (Fig. 2B, P<0.05). In addition, cell migration and invasion assays demonstrated that the miR-497 mimic reduced capacities of migration and invasion in TPC-1 and HTH83 cells (Fig. 2C, P<0.05). These results suggested that miR-497 functions as a tumor suppressor in PTC progression through inhibiting cell growth and metastasis.

**AKT3 is a direct target of miR-497 in PTC.** To investigate the mechanism of the tumor suppressive roles of miR-497 in PTC, a bioinformatics assay was used to predict putative targets of miR-497. Among numerous potential targets, AKT3 was focused on because of its regulation effects on multiple cancer-associated biological processes, such as cell proliferation, apoptosis, cell cycle procession, migration and invasion (25,26) (Fig. 3A).

To validate whether AKT3 is the right target gene of miR-497, a luciferase reporter assay was performed in HEK293T cells co-transfected with miR-497 mimics or miR-NC, and luciferase reporter vector. As presented in Fig. 3B, luciferase activity of the wild-type AKT3 3'UTR reporter gene was markedly reduced (P<0.05), whereas the luciferase activity of the mutant reporter gene was not affected. To further confirm this speculation, AKT3 expression in miR-497 mimics-transfected cells was detected at the mRNA and protein levels by RT-qPCR and western blotting. The results demonstrated that AKT3 was significantly decreased at mRNA (Fig. 3C, P<0.05) and protein (Fig. 3D, P<0.05) level in TPC-1 and HTH83 cells compared with miR-NC-transfected cells. Additionally, upregulation of miR-497 reduced p-AKT expression in TPC-1 and HTH83 cells, which may be caused by downregulation of p-AKT3 (Fig. 3D). These results demonstrated that AKT3 is a direct target gene of miR-497 in PTC.
AKT3 is reversely correlated with miR-497 expression in PTC. As AKT3 was identified to be a direct target of miR-497, it was hypothesized that miR-497 under-expression may contribute to the upregulation of AKT3 in PTC. To verify this hypothesis, AKT3 mRNA and protein expression levels in PTC tissues were determined. RT-qPCR and western blot analyses demonstrated that AKT3 was significantly upregulated in PTC tissues at both the mRNA (Fig. 4A, P<0.05) and protein (Fig. 4B, P<0.05) expression level compared with adjacent normal thyroid tissues. Furthermore, the negative correlation between miR-497 and AKT3 mRNA expression was confirmed by Pearson correlation analysis in PTC tissues (Fig. 4C, r=-0.5737 P<0.001). These findings suggested that the upregulation of AKT3 in PTC tissues may be caused by the downregulation of miR-497.

miR-497 inhibits the proliferation, migration and invasion of PTC via regulating AKT3 expression. AKT3 is a direct target of miR-497; therefore, it was speculated that enforced expression of miR-497-decreased cell growth and metastasis in PTC might be achieved by AKT3 knockdown. To confirm this, AKT3 siRNA was used to decrease AKT3 expression in TPC-1 and HTH83 cells (Fig. 5A, P<0.05). MTT and cell migration and invasion assays demonstrated that cell proliferation (Fig. 5B, P<0.05), migration and invasion (Fig. 5C, P<0.05) was suppressed in AKT3 siRNA-transfected TPC-1 and HTH83 cells compared with NC siRNA groups. Rescue experiments were also performed to examine whether the tumor suppressive roles of miR-497 in PTC were achieved by downregulation of AKT3. As presented in Fig. 5D, AKT3 was upregulated in TPC-1 and HTH83 cells after transfection with pcDNA3.1-AKT3 (P<0.05). Subsequently, rescue experiments demonstrated that upregulation of AKT3 almost completely reversed the inhibitory effects of miR-497 overexpression on proliferation, migration and invasion in both TPC-1 and HTH83 cells (Fig. 5E and F, P<0.05). These results suggested that miR-497 inhibited cell proliferation, migration and invasion of PTC, at least partially by regulating AKT3 expression.

Discussion
miR-497 has been demonstrated to be diversely expressed in several types of human cancer. For instance, previous studies have revealed that miR-497 is downregulated in breast cancer tissues and cell lines (27-29). Low expression levels of miR-497 indicate a poorer prognosis for patients with breast cancer (30). Wang et al (31) reported that miR-497 expression levels are reduced in colorectal cancer and correlate closely with clinical stage and lymph node metastases. A study by Luo et al (32) revealed that miR-497 expression is lowly expressed in both cervical cancer tissues and cell lines, and reduced miR-497 expression strongly correlates with lymph node metastases in patients with cervical cancer. Therefore, higher miR-497 expression may indicate a better prognosis. Zhang et al (33) demonstrated that expression levels of miR-497 were decreased in hepatocellular carcinoma, and was correlated with poor prognostic features. Zhao et al (34) demonstrated that miR-497 is downregulated in clear cell renal cell carcinoma, and correlated with tumor stage, histological grade and lymph node metastases. Low miR-497 expression may be a predictor of poor prognosis. Furthermore, miR-497 is expressed at low levels in non-small cell lung cancer (35), prostate cancer (36), pancreatic cancer (37), ovarian cancer (38), bladder cancer (39) and osteosarcoma (40). These findings suggested that miR-497 is frequently lowly expressed in human cancer and could be a therapeutic marker for its diagnosis and prognosis.

An increasing number of evidence has demonstrated that miR-497 serves key roles in the development and progression of multiple kinds of human cancer. In breast cancer, enforced expression of miR-497 suppresses tumor cell growth, migration, invasion, angiogenesis, epithelial mesenchymal transition,
and increases apoptosis by directly targeting multiple genes, such as RAF protocol-oncogene serine/threonine-protein kinase 1, G1/S-specific cyclin-D1, Bcl-2-like protein 2, cyclin...
E1, B-cell lymphoma l-2 and vascular endothelial growth factor (VEGF) 2 (27,28,31,41,42). In colorectal cancer, upregulation of miR-497 inhibits cell proliferation in vitro, reduces migration, invasion and metastasis in vitro and in vivo.
and enhances chemosensitivity to 5-fluorouracil treatment via blockade of kinase suppressor of Ras 1, insulin-like growth factor 1 receptor precursor (IGF-1R) and VEGFA (31,43,44). In cervical cancer, continued expression of miR-497 represses cell proliferation, colony-formation capacity and motility through regulating cyclin E1 and IGF-1R (32,45). In hepatocellular carcinoma, miR-497 overexpression inhibits cell colony formation, proliferation, angiogenesis and metastasis, and induces apoptosis by negative regulation of YAP1, IGF-1R, VEGFA and astrocyte elevated gene-1 (33,46,47). In non-small cell lung cancer, miR-497 re-expression attenuates cell proliferation, colony formation, growth, invasion and angiogenesis by downregulating hepatoma-derived growth factor, CCNE1, yes-associated protein 1 and VEGFA (35,48-50). Additionally, miR-497 has been reported to be involved in the occurrence and development of various other human cancers, including prostate cancer (36,51,52), pancreatic cancer (37,46), ovarian cancer (38,53,54), renal cancer (34), bladder cancer (39) and osteosarcoma (40,55,56). These findings strongly suggested that miR-497 may provide novel therapeutic target for the antitumor treatments.

As miR-497 is hypothesized to contribute to tumorigenesis and tumor development in PTC, the present study aimed to explore the mechanism underlying miR-497-inhibited induction of PTC cell growth and metastasis. Subsequently, an important molecular association between miR-497 and AKT3 was identified in PTC. Firstly, bioinformatics analysis predicted that AKT3 is a potential target gene of miR-497. Secondly, a luciferase reporter assay demonstrated that the 3’UTR of AKT3 could be directly targeted by miR-497. Thirdly, miR-497 decreased AKT3 expression at both the mRNA and protein level in PTC cells. AKT3 expression was upregulated in PTC tissues and inversely correlated with miR-497 expression. Finally, the roles of AKT3 knockdown were similar to the effects of miR-497 overexpression in PTC. Rescue experiments also demonstrated that miR-497 inhibited cell proliferation, migration and invasion of PTC, at least partially by negatively regulating AKT3. Identification of miR-497 target genes is pivotal for developing novel targeted therapies for the treatments of PTC.

AKT, a crucial factor of phosphoinositide 3-kinase/AKT signaling pathway, regulates various kinds of cellular processes such as cell proliferation, apoptosis, migration, invasion and metabolism (25,26,57,58). AKT3, a member of the AKT family, has been revealed to be upregulated in several kinds of human cancer, such as hepatocellular carcinoma (59), prostate cancer (60), pancreatic cancer (61,62), glioma (63) and breast cancer (64). Li et al (65) revealed that AKT3 was upregulated in PTC. Functional assays also demonstrated that AKT3 under-expression inhibits PTC cell growth and metastasis and induces apoptosis. The present study demonstrated that miR-497 directly targets AKT3 to suppress PTC cell proliferation, migration and invasion. Taken together, these data provided evidence to support that miR-497/AKT3 based targeted therapy could be a novel and efficient therapeutic strategy for patients with PTC. In conclusion, miR-497 functions as a tumor suppressor in PTC through inhibiting cell proliferation, migration and invasion. Notably, AKT3 was identified as a novel direct target of miR-497 in PTC. This novel miR-497/AKT3 signaling pathway may provide novel therapeutic tools for the treatment of patients with PTC.

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