

# MicroRNA-9-5p functions as a tumor suppressor in papillary thyroid cancer via targeting BRAF

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**Abstract.** MicroRNAs (miRNAs/miRs) are widely studied as key regulators of gene expression and are involved in various diseases by affecting the miRNA-mediated regulatory function. BRAF is an important oncogene in the regulation of cell proliferation and apoptosis. In the present study, reverse transcription-quantitative polymerase chain reaction was used to determine the expression levels of miR-9-5p and BRAF mRNA in patients with papillary thyroid cancer (PTC). Western blotting was used to detect BRAF protein level. A luciferase assay was used to verify the miR-9-5p target site in BRAF. Cell Counting Kit-8 and flow cytometry were used to assess cell proliferation, and apoptosis, respectively. In the present study, it was demonstrated that miR-9-5p is downregulated in malignant PTC. Using bioinformatics analysis, miR-9-5p was predicted to target the human BRAF 3'-untranslated region (3'-UTR). A dual-luciferase assay demonstrated that miR-9-5p downregulated BRAF expression by directly targeting its 3'-UTR. Mutations in the 3'-UTR of BRAF completely abolished its interaction with miR-9-5p. Expression of exogenous miRNA that mimics miR-9-5p decreased BRAF protein and mRNA levels, while suppression of endogenous miR-9-5p resulted in an increase in BRAF protein, and mRNA levels. Furthermore, regulation of miR-9-5p was observed to suppress the viability of PTC cells by inducing apoptosis. Consistently, downregulation of miR-9-5p promoted proliferation of PTC cells by inhibiting the apoptosis of cells. In conclusion, the present study demonstrated that miR-9-5p may perform an important role in PTC prognosis and therapy.

## Introduction

Globally, thyroid cancer is the eighth leading type of malignancy in women, accounting for 3% of all cancer types in women. The worldwide prevalence of thyroid cancer 15 years ago was 1.7%, which ranked 14th among all cancer types, and this value has now doubled. Each year, there are more incident cases of thyroid cancer compared with any other type of cancer (3.8%/year between 1992 and 2001) (1). Currently, it is estimated that there are 5-8 thyroid cancer cases/105 individuals each year in developed countries (2-5). In addition, the mortality rate of males with thyroid cancer has risen faster compared with other types of cancer, following adjustment of age (2.3% each year between 1992 and 2001), and papillary thyroid cancer (PTC) accounts for the majority of thyroid cancer types worldwide (5). In 2013, it was estimated that 60,000 cases would be newly diagnosed in the USA, and this number is increasing, which makes thyroid cancer an escalating and common disease (6,7). Although conventional options, including surgery and radioactive iodine are potent for PTC, they are not effective for undifferentiated (anaplastic) thyroid carcinoma (ATC), and advanced PTC, which are radioactive iodine-resistant (8).

A previous study reported an association between BRAF mutation, and the onset and development of PTC, as well as with the unfavorable prognosis of PTC (9). It has been demonstrated that mutations of the BRAF gene induce uncontrolled and persistent activation of kinase signaling pathways, causing over-proliferation, and differentiation into cancer cells (10). Notably, mutation of BRAF was identified in PTC progression of all stages. The presence of BRAF mutation in microcarcinoma or PTC at a very early stage indicates that this event may serve a role in the development of PTC tumorigenesis and susceptibility to another genetic variation, resulting in a more aggressive progression of PTC (9).

MicroRNAs (miRNAs) were identified at the turn of the 21st century, marking the milestone in cell biology of a new era. Changing the concept of the association between gene mRNAs and human disease has extended to contain those sequences in the residual ~90% of eukaryotic genomes involving generation of non-coding RNAs. miRNAs, which as meta-controllers of gene expression, are essential for the cellular alterations required for development. It is important to have detailed knowledge of the mechanisms involved in the

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regulation of mRNA expression, particularly considering that dysregulation of miRNAs is associated with several thyroid disorders, including, medullary thyroid carcinoma, follicular thyroid carcinoma and thyroid adenoma (11).

It has been previously demonstrated that miR-9-5p is differentially-expressed in PTC (12), and dysregulation of BRAF has also been reported to be involved in the molecular mechanism of PTC cell apoptosis (13,14). By searching the online miRNA database (www.targetscan.org), BRAF was revealed to be a virtual target of miR-9-5p with high similarity among species. In the present study, BRAF was validated as a target of miR-9-5p, and the involvement of miR-9-5p and BRAF in the development of PTC was verified.

## Materials and methods

**Sample collection.** A total of 25 female patients (age, 45±0.38 years; age range 44 years and 7 months-45 years and 4 months old) who were diagnosed with PTC and underwent surgery were recruited for the present study between February 2014 and February 2015 at the Department of Thyroid and Breast Surgery, The Second People's Hospital of Liaocheng (Liaocheng, China). An additional 25 benign thyroid nodule tissue samples without PTC were selected as controls. Subsequent to selecting patients, diagnosis was confirmed by two independent pathologists blinded to the clinical results. The protocol of the present study was approved by the Ethics and Research Committees of The Second People's Hospital of Liaocheng, and written informed consent was obtained from each patient prior to their involvement in the study.

**RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** The miRNeasy® Mini kit (TRIzol®; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to extract the total RNA from BCPAP cells, obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) in accordance with manufacturer's protocol. The RNeasy Mini spin column (Qiagen China Co., Ltd., Shanghai, China) was used to purify the target RNA according to the manufacturer's protocol, with a P260/280 nm ratio >1.8 considered acceptable. For subsequent experiments, the RNA was stored at -80°C in microcentrifuge tubes.

The ThermoScript RT-PCR system (Invitrogen; Thermo Fisher Scientific, Inc.) was used according to the manufacturer's protocol to reverse transcribe total RNA into cDNA. Fresh tissues were used to extract 1 mg RNA for the synthesis of cDNA with a mixture containing 5X cDNA synthesis buffer (250 mM Tris acetate pH 8.4), 40 nM magnesium acetate and 375 mM potassium acetate, and 10 mM dNTP mix, 15 U thermoscript transcriptase, 1 ml random hexamers (50 ng/ml), 0.1 M dithiothreitol and 40 U RNaseOUT (Invitrogen; Thermo Fisher Scientific, Inc.) in a final volume of 20 ml. The sequences used were as follows: miR-9-5p forward, 5'-CGAGCTCTGTGTGTGTGTGTGTGTG-3'; reverse, 5'-TTCCGCGGCCGCTATGGCCGACGTCGACGGGAATGGGGAAAGGGAA-3'; BRAF forward, 5'-GGCGGCAGCGCTGTGGCGGCG-3'; and reverse, 5'-CGTAGGGTCATACTCATCCAC-3'. The reaction was performed at 18°C for 25 min, 42°C for 30 min and 90°C for 7 min.

For qPCR, a SYBR-Green fluorophore (Applied Biosystems, Foster City, CA, USA) was used, and the thermo-cycling protocol was 10 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C. The expression of BRAF mRNA and miR-9-5p was calculated relative to the expression level of the endogenous control,  $\beta$ -actin. The  $2^{-\Delta\Delta C_q}$  method (15) was used to analyze the relative quantification of BRAF mRNA. Expression of miR-9-5p was normalized to U6, the sequence of primer for U6 is: Forward 5'-CTCGCTTCGCGAGCACCA-3' and Reverse 5'-AACGCTTCACGAATTTGCGT-3'. Every experiment was performed at least three times.

**Cell culture and transfection.** Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.), supplemented with 50 mg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA) and 50 mg/ml penicillin was used to maintain BCPAP cells in an atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C. The BCPAP cells were seeded on 6-well plates containing growth medium and antibiotics at a density of 3-6x10<sup>5</sup> cells/well for 12 h prior to transfection. When 80% confluence was achieved, 100 pM miR-9-5p mimics; CGAGCTCTGTGTGTGTGTGTGTGTGTG-3'; miR-9-5p inhibitors; 5'-TTCCGCGGCCGCTATGGCCGACGTCGACGGGAATGGGGAAAGGGAA-3', or BRAF small interfering RNA; 5'-GGCGCTCCCTTCCCCCTCCCCTT-3' (siRNA; all Invitrogen; Thermo Fisher Scientific, Inc.) were transfected into the cells using 2  $\mu$ l Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at 37°C. The time interval between the completion of transfection and the use of the transfected cells was 4 h. The sequence of siRNA was: 5'-AAGUGGCAUGGUGAUGUGGCA-3', and the final concentration used for transfection was 20 pmol.

**Vector construction and mutagenesis.** In order to determine miRNA targeting of the 3'-UTR region of BRAF, the full length of the BRAF 3'-UTR was amplified and cloned into a pmir-REPORT luciferase vector (Promega Corporation, Madison, WI, USA). DNA was extracted from the tissue samples using the PicoPure™ DNA Extraction Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), and treated with DNA polymerase (Promega Corporation). The sequences of the primers were as follows: BRAF forward, 5'-GGCGGCAGCGCTGTGGCGGCG-3'; and reverse, 5'-CGTAGGGTCATACTCATCCAC-3'. The temperature protocol was as follows: 95°C for 3 min, followed by 30 cycles of 94°C for 40 sec, 56°C for 35 sec and final extension at 72°C for 60 sec. The Vybrant™ MTT Cell Proliferation Assay kit (Thermo Fisher Scientific, Inc.) was used to generate the mutated vector by replacing the miR-9-5p binding site nucleotides, according to the manufacturer's protocol.

**Cell proliferation assay.** An MTT assay was performed to analyze the proliferation of BCPAP cells in accordance with the manufacturer's protocol (Thermo Fisher Scientific, Inc.). DMEM supplemented with 10% FBS was used to incubate the BCPAP cells on 96-well plates at a final concentration of 3x10<sup>4</sup> cells/ml. MTT (10  $\mu$ l; Thermo Fisher Scientific, Inc.) was then added to each well, and the culture medium was incubated for 1, 2 and 3 days at 37°C. Subsequent to culturing

for an additional 48 h, 1% dimethyl sulfoxide was used to dissolve the formazan crystals, a blood counting chamber was used to count the number of cells, and an ELISA plate reader was used to analyze the proliferation of BCPAP cells based on the absorbance, which was read at 440 nm.

**Luciferase assay.** The luciferase reaction mixture included  $1 \times 10^6$  BCPAP cells, 1  $\mu$ g of a *Renilla* luciferase expression construct pRL-TK (Promega Corporation), 1  $\mu$ g p-BRAF (wild-type or mutant) plasmid and 50 pmol miR-9-5p mimics (or control). The dual-luciferase assay system (Promega Corporation) was used to detect the luciferase activity according to *Renilla* luciferase activity at 36 h post-transfection, according to the manufacturer's protocol. Each experiment was performed at least three times, and transfection was performed with Lipofectamine 2000<sup>®</sup> (Invitrogen; Thermo Fisher Scientific, Inc.).

**Western blot analysis.** For analysis of the expression of BRAF mRNA and miR-9-5p from tissue samples and BCPAP cells, radio immunoprecipitation assay lysis buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) containing protease inhibitors (Roche Applied Science, Madison, WI, USA), 5 g/l sodium deoxycholate, 0.2 g/l Na<sub>3</sub>N, 10 ml/l NP-40, 150 mmol/l NaCl 100  $\mu$ g/ml, 0.1 g/l SDS, phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml aprotinin and 50 mmol/l Tris-HCl (pH 8.5) was used to lyse the BCPAP cells following transfection, according to manufacturer's protocol, followed by two washes with PBS (Invitrogen; Thermo Fisher Scientific, Inc.). Protein determination was performed using a BCA assay (Beyotime Institute of Biotechnology, Haimen, China). SDS-PAGE (12%) was used to purify the target proteins, and proteins (35  $\mu$ g/lane) were then transferred to a polyvinylidene fluoride membrane (GE Healthcare, Chicago, IL, USA). Tween-20 or TBS with 5% non-fat milk (Sigma-Aldrich; Merck KGaA) was used to block the membrane for 2 h at room temperature. The primary anti-BRAF antibodies in TBS buffer (dilution, 1:1,000; cat. no., sc-136263; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used to detect the target protein via incubation for 12 h at 4°C. Subsequently, membranes were washed twice with PBS, followed by incubation with horseradish peroxidase-conjugated secondary antibody (dilution, 1:3,000; cat. no. 7075S Cell Signaling Technologies, Inc., Danvers, MA, USA) for another 2 h at 25°C. Prior to film exposure, a peroxidase substrate was used to enhance chemiluminescence (Thermo Fisher Scientific, Inc.). Amersham ECL Prime Western Blotting Detection reagent (cat. no. RPN2236; GE Healthcare, Chicago, IL, USA) was used to achieve visualization of the relative enrichment of proteins. Bio-Rad Chemi-DocXRS (Bio-Rad Laboratories, Hercules, CA, USA) was utilized to visualize band of target protein.

**Apoptosis analysis.** In order to analyze apoptosis, 7-amino-actinomycin D (7-AAD) was used to dual stain the BCPAP cells after transfecting for 72 h, and the Annexin V-fluorescein isothiocyanate (FITC)/7-AAD kit (Beckman Coulter, Inc., Brea, CA, USA) was used to detect the Annexin V-FITC, according to the manufacturer's protocol. Flow cytometry (Cell Lab Quanta SC; Beckman Coulter, Inc.) with 585/42 nm (PI) and 530/30 nm (FITC) emission

filters was used to analyze the apoptosis of cells after staining cells according to the manufacturer's protocol. FCAP Array Software v3.0 was used for analysis (Cell Lab Quanta SC; Beckman Coulter, Inc.). All experiments were performed in triplicate.

**Statistical analysis.** All values are presented as the mean  $\pm$  standard deviation. For the bioinformatics analysis, computational algorithms (www.targetscan.org; access date 02/02/2015) were used to predict putative miR-9-5p targets. Fisher's exact test was used to analyze the comparisons of categorical variables. Comparisons among the continuous variables were performed using Spearman's Rank Correlation and Sign test.  $P < 0.05$  was considered to indicate a statistically significant difference. StatView Software (v.5, SAS Institute, Inc., Cary, NC, USA) was used to analyze the statistical analysis.

## Results

**miR-9-5p is downregulated in malignant PTC.** To identify miRNAs regulating malignant PTC, miRNA expression levels were analyzed between PTC and benign thyroid nodules. miR-9-5p was consistently downregulated in malignant PTC compared with benign tumor samples ( $P < 0.05$  vs. Thyroid nodule tissue samples; Fig. 1). Therefore, the present study focused on the function of miR-9-5p, which was hypothesized to distinguish the malignant tumors from the benign ones.

**BRAF expression transcripts are targeted by miR-9-5p.** One of the biggest challenges in studying miRNAs is to identify target genes and associate their downregulation with cellular properties. Computational algorithms (www.targetscan.org) have been used to predict putative miR-9-5p targets based on complementarity to the 3'-UTR of the target mRNA. Through bioinformatics analysis, miR-9-5p was predicted to target the human BRAF 3'-UTR (Fig. 2). To investigate whether miR-9-5p is a real BRAF regulator, the following experiments were performed. Firstly, luciferase reporter vectors containing a wild-type or mutation BRAF 3'-UTR sequence at the downstream of luciferase were constructed (Fig. 2). miR-9-5p mimics and corresponding reporter vectors were co-transfected into the BCPAP cell line. A dual-luciferase assay was performed at 48 h post-transfection. The results of the dual-luciferase assay revealed that when the reporter vector contained a wild-type BRAF 3'-UTR, miR-9-5p significantly suppressed the luciferase expression compared with the scramble control group ( $P < 0.05$  vs. scramble control) (Fig. 3). Consistently, mutation of the target region completely abolished this observation, returning levels of luciferase activity similar to that of the scramble control group (Fig. 3).

**BRAF expression is upregulated in PTC.** It was hypothesized that the reduction of miR-9-5p leads to the increase of BRAF expression level in malignant papillary thyroid tumors. To support this hypothesis, RT-qPCR and western blot analysis was used to determine the expression level of BRAF mRNA, and protein in the two groups (Fig. 4). The results demonstrated that BRAF mRNA and protein expression was



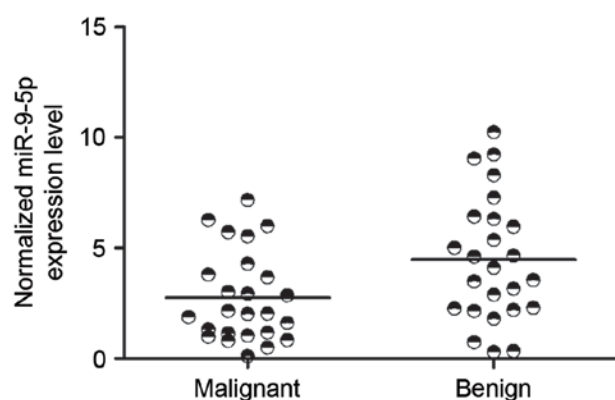


Figure 1. miR-9-5p is downregulated in malignant PTC. Expression levels of miR-9-5p were detected in RNA samples extracted from malignant (n=25) or benign (n=25) PTC tissues. Expression of miR-9-5p was normalized to U6. miR-9-5p, microRNA-9-5p; PTC, papillary thyroid cancer.

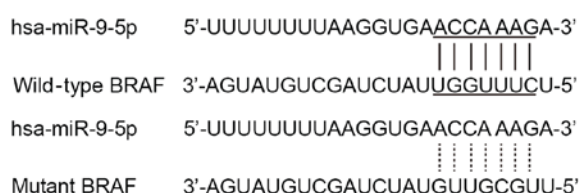


Figure 2. Schematic illustrating the predicted miR-9-5p binding sites in the 3'-untranslated region of BRAF mRNA. BRAF mutations were constructed by destroying the base pairing between miRNA and its target in seed regions. miR-9-5p, microRNA-9-5p.

upregulated in malignant papillary thyroid tumor compared with that in benign samples ( $P < 0.05$  vs. benign tumor samples).

**Effect of miR-9-5p upregulation or downregulation on the expression of BRAF.** To determine whether miR-9-5p disrupts BRAF expression in BCPAP cells, miR-9-5p mimics were transfected into BCPAP cells. Scramble served as a negative control, while BRAF siRNA served as a positive control. The data revealed that BRAF mRNA and protein level could be suppressed by miR-9-5p mimic ( $P < 0.05$  vs. scramble control) (Fig. 5). Furthermore, similar experiments were performed using miR-9-5p inhibitor treatment and the decrease in miR-9-5p increased BRAF mRNA and protein levels ( $P < 0.05$  vs. scramble control) (Fig. 5). These results demonstrated that miR-9-5p targets BRAF 3'-UTR in a direct manner, therefore suppressing the expression of BRAF.

**MiR-9-5p affects cell proliferation and apoptosis by regulating BRAF expression.** BRAF performs an important role in regulating cell proliferation, and high levels of BRAF may promote proliferation of malignant cells by suppressing apoptosis (16). To verify this, miR-9-5p mimics and inhibitor were introduced into PTC cells, with scramble serving as a negative control. As presented in Fig. 6, miR-9-5p and BRAF siRNA suppressed the viability of PTC cells by inducing apoptosis ( $P < 0.05$  vs. scramble control), and consistently, downregulation of miR-9-5p promoted proliferation of PTC cells by inhibiting cell apoptosis ( $P < 0.05$  vs. scramble control).

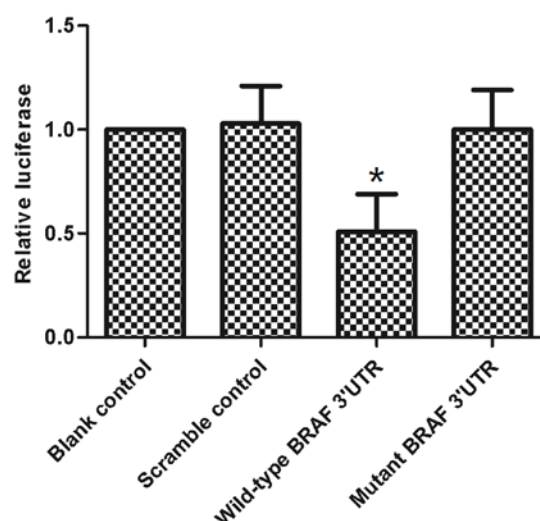


Figure 3. Luciferase activity of reporter genes containing a wild BRAF and mutant 3'-UTRs. PTC cells were co-transfected with miR-9-5p mimic and indicated BRAF 3'-UTR-luciferase reporter. Blank and scramble served as negative controls. Luciferase activity was assayed 48 h later. All data are presented as the mean  $\pm$  standard deviation, normalized to the blank control of three independent experiments. miR-9-5p, microRNA-9-5p; 3'-UTR, 3'-untranslated region. \* $P < 0.05$  compared with the blank controls.

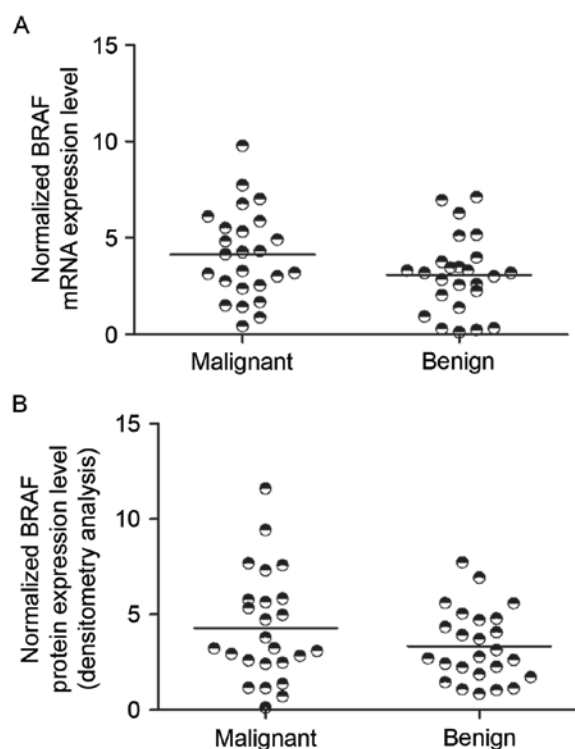


Figure 4. BRAF mRNA and protein levels were upregulated in malignant papillary thyroid tumor. The expression levels of (A) BRAF mRNA and (B) BRAF protein were compared between malignant (n=25), and benign (n=25) papillary thyroid tumors.

## Discussion

MiRNAs have been reported to be functionally involved in the regulation of biological processes, and may function as oncogenes or tumor suppressors in the development of

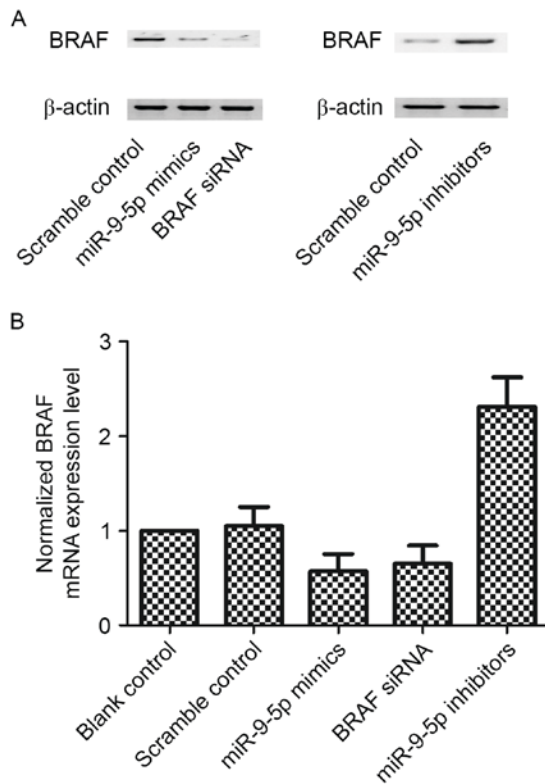


Figure 5. BRAF protein level and mRNA level can be inhibited by miR-9-5p. miR-9-5p mimics or inhibitor were transfected into BCPAP cells. (A) Western blot analysis was performed to analyze BRAF protein level. (B) Relative expression levels of BRAF mRNA were determined by reverse transcription-quantitative polymerase chain reaction. Blank and scramble controls served as negative controls. BRAF siRNA served as a positive control. Values are presented as the mean  $\pm$  standard deviation. miR-9-5p, microRNA-9-5p; siRNA, small interfering RNA.

various malignancies (10). The invasiveness of mesothelial cells (MCs) is markedly reduced when the levels of miR-9-5p are increased, which reveals a mechanism involving prevention of build-up of myofibroblasts derived from MCs in the submesothelial compact zone of the peritoneum, thereby preventing it from initiating the process of fibrosis (17). Notably, over-expression of miR-9-5p also reduced NADPH oxidase 4 levels induced by transforming growth factor- $\beta$  (TGF- $\beta$ )1 and TGF- $\beta$  receptor 2, representing a possible mechanism involving the anti-fibrotic effect of miR-9-5p in pleural mesothelial cells (18). The level of miR-9-5p in human non-epithelial cells was revealed to be higher compared with that in epithelial MCs, which demonstrates that miR-9-5p protects against the development of pulmonary fibrosis (19). In the present study, miRNA expression levels were analyzed between PTC and benign thyroid nodule tissue samples. MiR-9-5p was consistently downregulated in PTC compared with the normal control.

To identify the downstream effector of miR-9-5p, bioinformatics analysis was performed and revealed that miR-9-5p was predicted to target the human BRAF 3'-UTR. Furthermore, a luciferase assay was performed, demonstrating that when the reporter vector contained a wild-type BRAF 3'-UTR, miR-9-5p significantly suppressed the luciferase expression. Consistent with this, mutation of target regions completely abolished this interaction. In addition, miR-9-5p mimics were

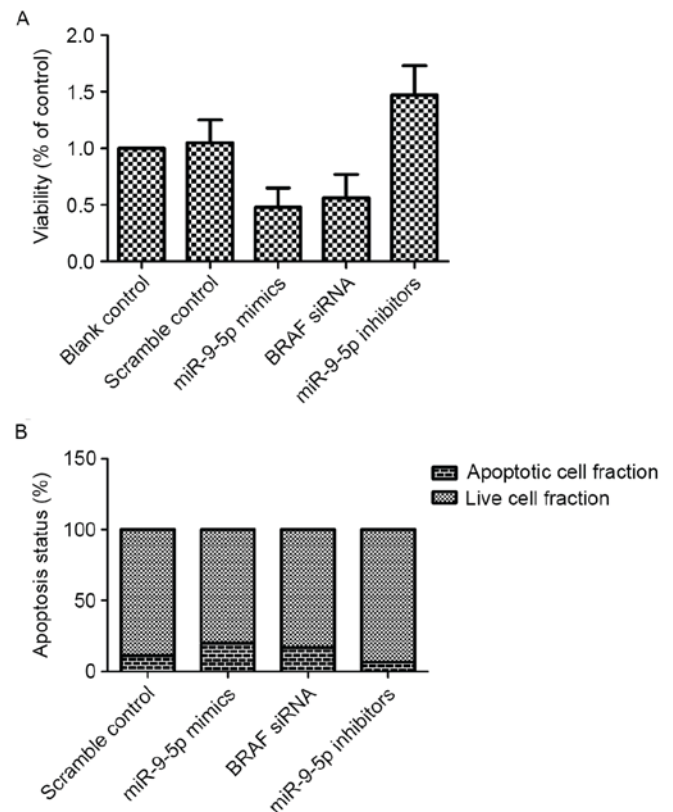


Figure 6. Effect of miR-9-5p on viability and apoptosis in papillary thyroid cells. (A) miR-9-5p mimics or inhibitors were transfected into papillary thyroid cells. The Cell Counting Kit-8 assay was performed to determine the effect of miR-9-5p on papillary thyroid cell proliferation. Blank or scramble served as negative controls. (A) siRNA against BRAF served as a positive control. (B) miR-9-5p mimics or inhibitors were transfected into papillary thyroid cells. An apoptosis assay was performed to determine the effect of miR-9-5p on cell apoptosis. Scramble served as negative control. siRNA against BRAF served as a positive control. miR-9-5p, microRNA-9-5p; siRNA, small interfering RNA.

transfected into BCPAP cells, and scramble served as a negative control, while BRAF siRNA served as a positive control. The data demonstrated that BRAF mRNA and protein levels were suppressed by miR-9-5p mimic. Furthermore, similar experiments were performed using miR-9-5p inhibitor treatment, and the decrease of miR-9-5p increased BRAF mRNA and protein levels. These results confirmed that miR-9-5p targets the BRAF 3'-UTR directly, and therefore suppresses the expression of BRAF.

The cascade of RAF-mitogen-activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK) protein kinases serves a major role in transforming extracellular mitogenic signals to cell proliferation, as well as other cellular functions (20,21). The family of RAF Ser/Thr kinases includes A-, B- and C-RAF, which have similar domain structures. It has been demonstrated that hetero- and homo-dimerization of RAF family proteins causes an essential change in MEK phosphorylation and activation (kinase regulated by extracellular signal/protein kinase activated by mitogen), and consequently ERK (kinase regulated by extracellular signal) responding to the activation of RAS (22). Kinase suppressors of RAS proteins primarily serve as scaffolds of the signaling cascade and are also homologs of RAF family members, tethering

ERKs, MEKs and RAFs together. In human cancer, the mutations are commonly present in the RAF-MEK-ERK signaling pathway (23). A total of ~6% of all human cancers and 50% of melanomas exhibit BRAF mutations (24). In >90% of BRAF mutations, there is a single base substitution present in a codon of the kinase domain, contributing to V600E amino acid alteration, constitutive active BRAF protein kinase, and subsequently downstream signaling of MEK-ERK (21). In the present study, it was hypothesized that the reduction of miR-9-5p leads to the increase of BRAF expression level in malignant papillary thyroid tumors. To test this hypothesis, RT-qPCR was used to determine the expression level of BRAF mRNA in two groups, and it was revealed that BRAF expression is upregulated and miR-9-5p is downregulated in malignant papillary thyroid tumor.

A transversion point mutation (1799T >A) in the BRAF gene has been observed in 20-40% of ATCs and 45% of PTCs, leading to a valine to glutamate substitution at amino acid 600 of the protein termed BRAFV600E, and finally constitutive activation of kinase activity (25,26). It is evident that BRAF mutants in tumors are not always a signal of clinically aggressive thyroid cancer in patients, although it is true that BRAFV600E has an important role in tumor behavior (27,28). Other immune factors and gene pathways are either known or putatively unknown to interact with mutant BRAF signaling, which involves in the formation of aggressive features in thyroid tumors (29). Mutations that affect the phosphoinositide 3-kinase (PI3K)-protein kinase B (AKT) pathway and tumor suppressor p53 are among other genetic events revealed to activate dedifferentiation, and tumor progression (27). The majority of ATCs exhibit inactive p53 (30). The inactivation of the tumor suppressor phosphatase and tensin homolog is less prevalent compared with mutations of p53, which are observed in ~15% of cases of ATC and contributes to the activation of the PI3K-AKT signaling pathway (31). Furthermore, efforts will be made to identify other putative relevant drivers of mutations and signaling pathways, including those made by The Cancer Genome Atlas (<http://tcga-data.nci.nih.gov/tcga/>). Thyroid cancer progression can be well studied using mouse models. Using orthotopic and genetically engineered approaches that have been designed more recently, the mouse models of PTC, and the more aggressive and lethal types of thyroid cancer, ATC, have been established (32). There are strengths and drawbacks to each of these approaches. A previous study identified an association between mortality rate and BRAF status, previously regarded as additional information to traditional factors of prognosis (33). The BRAF mutation may also be a potential marker for predicting the response to targeted treatments considering the availability of existing targeted treatments, including a BRAF inhibitor (34). However, there remains debate about the role of the BRAF mutation in prognosis, since a prognostic role for the BRAF mutation has not been identified in numerous studies (35,36). In the present study, regulation of miR-9-5p was found to suppress the viability of PTC cells by inducing apoptosis, and consistently, downregulation of miR-9-5p promoted proliferation of PTC cells by inhibiting apoptosis of the cells.

In conclusion, the findings of the present study revealed that BRAF is a direct target of miR-9-5p, and the miR-9-5p/BRAF signaling pathway may perform an important role in the

tumorigenesis of PTC. Furthermore, miR-9-5p/BRAF may be a therapeutic target in the treatment of PTC.

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## Availability of data and materials

The datasets generated and analyzed in the present study are included in this published article.

## Authors' contributions

FG was responsible for the planning of the present study, data collection, analysis and interpretation and preparation of the manuscript. XH also participated in data collection, analysis and interpretation and was responsible for the collection of funds. QS was responsible for data analysis and interpretation, preparation of the manuscript and literature analysis.

## Ethics and consent to participate

The protocol of the present study was approved by the Ethics and Research Committees of The Second People's Hospital of Liaocheng, and written informed consent was obtained from each patient prior to their involvement in the study.

## Patient consent for publication

The study participants provided consent for the data in the present study to be published.

## Competing interests

The authors declare that they have no competing interests.

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