Oncology Research, Vol. 25, pp. 1215–1222
Printed in the USA.

0965-0407/17 $90.00 + .00
DOI: https://doi.org/10.3727/096504016X14791715355957
E-ISSN 1555-3906
www.cognizantcommunication.com

Upregulated miR-9-3p Promotes Cell Growth and Inhibits Apoptosis in Medullary Thyroid Carcinoma by Targeting BLCAP

Yangjing Chen, Shaoqiang Zhang, Ruimin Zhao, Qian Zhao, and Ting Zhang

Department of Otolaryngology Head and Neck Surgery,
The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, P.R. China

Medullary thyroid carcinoma (MTC) is a neuroendocrine cancer derived from parafollicular C cells in the thyroid gland. It has great interest as a research focus because of its unusual genetic, clinical, and prognostic characteristics. However, the pathogenesis in MTC is not completely clear. We investigated the role of miR-9-3p and bladder cancer-associated protein (BLCAP) in MTC TT cells. First, miR-9-3p expression was upregulated in human MTC tissues and TT cells and compared to the control by RT-PCR. Flow cytometric analysis indicated that the cell cycle progression in TT cells was significantly inhibited by the miR-9-3p inhibitor but was increased by the miR-9-3p mimic. On the contrary, the apoptosis of TT cells was significantly increased by the miR-9-3p inhibitor and suppressed by the miR-9-3p mimic. A similar change pattern was observed in the expression of apoptosis-regulated protein caspase 3 induced by the miR-9-3p mimic or inhibitor in TT cells. We then identified that BLCAP is a target of miR-9-3p by bioinformatic prediction and luciferase reporter assay. The expression of BLCAP was also significantly downregulated by the miR-9-3p mimic while being upregulated by the miR-9-3p inhibitor in TT cells. Furthermore, we confirmed that the inhibited apoptosis of TT cells induced by the miR-9-3p mimic was enhanced by BLCAP overexpression. The levels of apoptosis were strongly decreased by BLCAP silencing in TT cells, which were not further influenced by the miR-9-3p inhibitor. In summary, upregulated miR-9-3p has a positive role in human MTC progression by regulating the growth and apoptosis of cancer cells via targeting BLCAP. This might represent a possible diagnosis or therapeutic target for MTC.

Key words: Medullary thyroid carcinoma (MTC); miR-9-3p; Bladder cancer-associated protein (BLCAP); Cell growth; Apoptosis

INTRODUCTION

Medullary thyroid carcinoma (MTC) is a neuroendocrine cancer derived from parafollicular C cells in the thyroid gland and accounts for only 5%–8% of all thyroid carcinomas around the world[1]. Despite its uncommonness, MTC is still of great interest as a research focus because of its unusual genetic, clinical, and prognostic characteristics[2]. There are two predominant types, sporadic MTC and hereditary MTC, according to the difference in pathogenesis characteristics. About 75% of MTC cases are sporadic and 25% are hereditary. The activated mutations in rearranged during transfection (RET) proto-oncogenes have been recognized as a major pathogenesis in the progression of MTC[3]. MTC is the result of a complicated process by which the normal protein conformation in thyroid parafollicular cells is induced by oncogenic factors to be altered, resulting in the excessive proliferation of parafollicular cells[4,5]. According to the difference in patients' symptoms, hereditary MTC can also be classified into multiple endocrine neoplasia type 2A (MEN2A), MEN2B, and a family of nonmultiple endocrine adenomatous MTC (FMTC). MEN2A accounts for 80% of hereditary MTCs, usually coexisting with pheochromocytoma and parathyroid hyperplasia in patients. MEN2B is the type with the highest degree of malignancy in hereditary MTC and is characteristic of mucosal multiple neuroma with MTC and/or adrenal pheochromocytoma. FMTC is identified to be a variation of MEN2B[6,7]. Despite aggressive surgery, there are still problems related to the efficiency of therapy and clinical outcomes. Thus, further understanding of the pathogenesis of MTC is necessary to improve the diagnosis and treatment of MTC.

Aberrant expression of miRNAs has been reported in many pathological processes and has been considered

Address correspondence to Shaoqiang Zhang, Department of Otolaryngology Head and Neck Surgery,
The First Affiliated Hospital of Xi'an Jiaotong University, 277 Yanta West Road, Xi'an 710061, Shaanxi, P.R. China.
Tel: +86-029-85323965; Fax: +86-029-85323965; E-mail address: zhshao_qiang@126.com

to be implicated in the progression of human diseases[8]. miRNAs are small single-stranded noncoding RNA molecules that play the role of endogenous regulator in gene expression[9]. Most miRNAs exert their function by binding to target mRNAs to form a comprehensive regulatory network, which is involved in various biological processes, including cell proliferation, death, differentiation, and others[10,11].

In thyroid malignancies as well as MTC, dysregulation of miRNA expression has been widely reported, including miR-146b, miR-38, miR-183, miR-199b, miR-323, miR-221, miR-127, miR-370, miR-21, miR-183, miR-154, miR-375, and miR-224[12–14]. These miRNAs have been recognized to be associated with carcinogenesis. In terms of miR-9-3p, previously named miR-9*, it has been found to be abnormally expressed and have a regulatory role in many diseases, such as trimethyltin-induced neurotoxicity, Huntington's disease, glioma, primary brain tumors, and others[15–18]. A previous study reports that miR-9-3p is significantly upregulated in MTC[13]. Nevertheless, the role of miR-9-3p in the progression of MTC is still unclear.

Bladder cancer-associated protein (BLCAP), also known as bladder cancer 10-kDa protein (BC10), is a conserved transmembrane protein with 87 amino acids and two transmembrane regions (TMs)[19]. The BLCAP gene was originally considered to be a novel tumor suppressor in human bladder carcinoma. It has been identified in multiple cancers, including HeLa cells, human Ewing's sarcoma, and tongue carcinoma[20–22]. In the present study, we demonstrated the potential role of miR-9-3p and BLCAP in MTC. miR-9-3p expression was increased in MTC tissues and played a regulatory role in thyroid parafollicular cell motility via directly targeting BLCAP. Our findings indicate a novel role for miR-9-3p in MTC pathogenesis, which might represent a possible diagnosis or therapeutic target for MTC.

MATERIALS AND METHODS

Patients and Tissue Samples

Frozen biopsy specimens of tumor samples were collected from 12 patients with MTC undergoing surgery at the First Affiliated Hospital of Xi'an Jiaotong University (P.R. China) and eight nontumor donors. The study was approved by the ethics committee of the First Affiliated Hospital of Xi'an Jiaotong University. All patients gave their informed consent.

Cell Culture

The human MTC cell line TT, as well as normal thyroid epithelial cells, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). TT cells were maintained in RPMI-1640 medium supplemented with 16% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO_2 atmosphere. Normal thyroid epithelial cells were cultured in DMEM containing 10% FBS in a humidified atmosphere of 5% CO_2 at 37°C.

Quantitative RT-PCR

TRIzol reagent (Invitrogen) was used to extract the total RNA from tissues and cells. RT-PCR for miR-9-3p was performed using MicroRNA First-Strand Synthesis and miRNA Quantitation kits (Takara, Dalian, P.R. China) according to the manufacturer's protocol. RT-PCR for BLCAP was prepared using the CellAmp Direct RNA Prep Kit for qPCR and a Protein Analysis kit (Takara). U6 and GAPDH were used as the internal control.

Western Blotting

Total proteins were isolated from tissues and cells and separated by SDS-PAGE (Invitrogen). Western blot assay was then performed according to the manufacturer's protocol. Primary antibodies [rabbit anti-BLCAP (1:1,000 dilution) and mouse anti-β-actin (1:3,000 dilution); Abcam, Cambridge, MA, USA] and specific secondary antibodies were used. Bands were visualized by ECL (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Cell Transfection

For cell transfection, 100 nM miR-9-3p mimic, inhibitor, and negative control miRNA (NC; RiboBio, Guangzhou, P.R. China) were transfected into cells using Lipofectamine 2000 (Invitrogen) twice for 48 h. Transfection of Bcl2l2 siRNA was also performed using Lipofectamine 2000.

The open reading frame (ORF) of BLCAP was cloned into the pAdTrack-CMV vector (Clontech Laboratories, Mountain View, CA, USA). The adenovirus particles containing pAd-BLCAP were obtained according to the manufacturer's protocol. Cells were infected with adenoviruses for 48 h without apparent cytotoxicity.

Cell Apoptosis

Cell apoptosis assay was performed using the Annexin-V-Fluorescein Isothiocyanate Kit (Immunotech, Marseille, France) according to the manufacturer's protocol. Briefly, after being cultured in serum-free DMEM for 16 h, cells were harvested and resuspended. Cell suspensions were then stained with 0.5 μg/ml annexin V-fluorescein isothiocyanate and 0.6 μg/ml propidium iodide (PI), followed by flow cytometry analysis (FACSCalibur; BD Biosciences, San Jose, CA, USA).

Cell Cycle Assay

Cell cycle assay was performed as described previously[23,24]. After transfection for 48 h, cells were harvested

and suspended in PBS, and then fixed in 70% ethanol overnight. Ethanol was then replaced with RNase A. The suspension was incubated at 37°C and stained with PI in the dark for 30 min, followed by flow cytometry (FACSCalibur).

Luciferase Reporter Assay

Luciferase reporter analysis was performed as described previously[23]. In brief, the 3′-UTR of BLCAP was cloned into the pGL3 luciferase vector (Promega, Madison, WI, USA). Site-directed mutagenesis was introduced into the predicted miR-9-3p binding site in BLCAP 3′-UTR via QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA, USA). Recombinant pGL3 vector was cotransfected with miR-9-3p mimic into cultured cells (5×10^5 cells/well) using Lipofectamine 2000. After 36 h, luciferase activity was analyzed using the dual luciferase assay (Promega).

Statistical Analysis

Experiments were performed at least three times. Data are expressed as mean±SEM. The differences between groups were analyzed by GraphPad Prism 6 (La Jolla, CA, USA). A value of $p<0.05$ was considered as statistically significant.

RESULTS

miR-9-3p Is Upregulated in Human MTC Tissues and TT Cells

To evaluate the potential role of miR-9-3p in human MTC, we investigated the level of miR-9-3p expression in MTC tumor tissues from patients. Results showed that the expression of miR-9-3p was significantly upregulated in MTC tumor tissues compared to that in normal tissues (Fig. 1A). We further identified the expression pattern of miR-9-3p in the representative human MTC cell line TT cells. The level of miR-9-3p was significantly increased in TT cells when compared to the normal thyroid epithelial cells (Fig. 1B). These results indicate that the dysregulation of miR-9-3p expression may have a regulatory role in the tumorigenesis of human MTC.

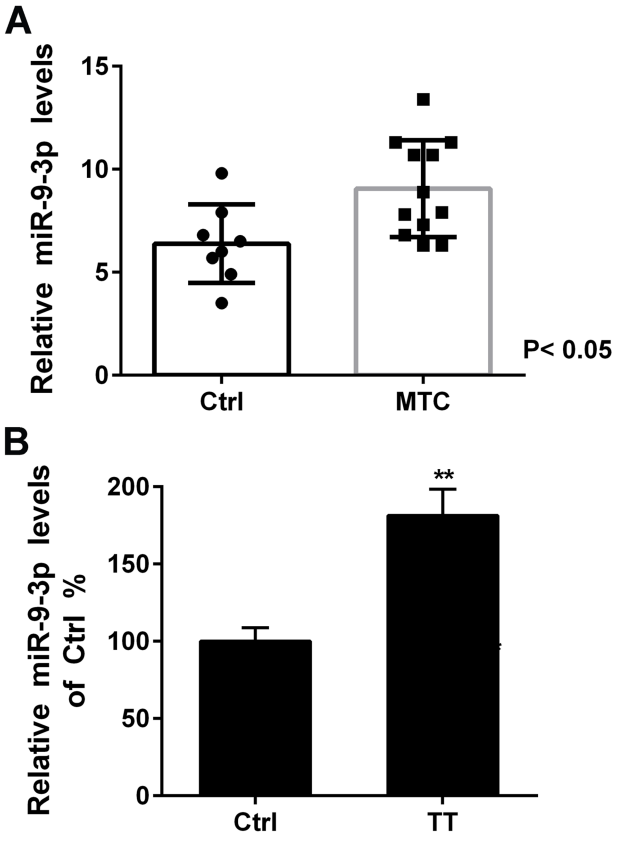

Figure 1. The expression of miR-9-3p in human MTC tissues and TT cells. (A) The levels of miR-9-3p in human MTC tissues and normal tissues (Ctrl) were tested by RT-PCR. (B) The expression of miR-9-3p in human MTC cell line TT cells as well as the normal thyroid epithelial cells (Ctrl) was tested by RT-PCR. $^{**}p<0.01$ versus Ctrl.

miR-9-3p Promotes Cell Cycle Progression in TT Cells

To further clarify the possible effect of upregulated miR-9-3p levels on human TT cells, we used the specific mimic or inhibitor for miR-9-3p in subsequent experiments. RT-PCR assay was performed to evaluate the efficiency of specific miR-9-3p mimic or inhibitor. miR-9-3p expression was obviously enhanced by the mimic while being inhibited by the specific inhibitor in TT cells, indicating the sufficient efficiency of the miR-9-3p mimic and inhibitor (Fig. 2A).

Cell cycle assay was then performed via flow cytometric analysis, as described previously. Results showed that the percentage of cells at the G_0/G_1 phase was dramatically lower, while the percentage of cells at the S phase was much higher in the miR-9-3p mimic group than that in the control group (Fig. 2B). On the contrary, the inhibition of endogenous miR-9-3p strongly suppressed cell cycle progression, in which the percentage of cells at the G_0/G_1 phase was higher and those at the S phase were lower (Fig. 2B). These data suggest that miR-9-3p could promote cell cycle progression in TT cells.

miR-9-3p Inhibits Apoptosis in TT Cells

We then investigated the effect of miR-9-3p on human TT cell apoptosis. The level of apoptosis of cultured TT cells was dramatically reduced by the miR-9-3p mimic when compared with the control (Fig. 3A). On the contrary, miR-9-3p inhibition significantly increased the rate of apoptosis in TT cells when compared to the control. A similar pattern change was observed in the levels of caspase 3, an apoptosis-regulated protein, in TT cells. We further measured the expression of cleaved caspase 3

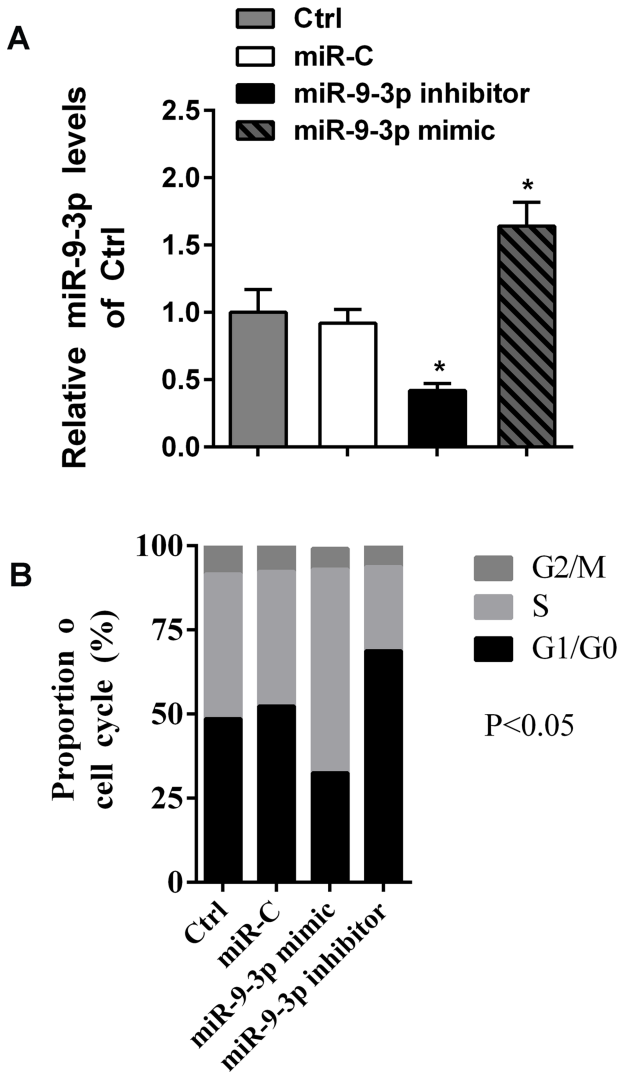

Figure 2. Effects of miR-9-3p on cell cycle progression in TT cells. TT cells were cultured (Ctrl), followed by transfection with the miR-9-3p mimic or inhibitor, as well as the negative control miRNA (miR-C). (A) The expression of miR-9-3p was measured using RT-PCR in TT cells transfected with the specific mimic, inhibitor, or negative miRNA (miR-C). (B) Cell cycle assay was then performed using flow cytometric analysis. *$p < 0.05$ versus Ctrl.

in TT cells by Western blot assay. The expression level of cleaved caspase 3 was reduced by miR-9-3p overexpression but was enhanced by miR-9-3p suppression (Fig. 3B). These results reveal that miR-9-3p plays a negative role in the regulation of MTC apoptosis.

BLCAP Is the Direct Target of miR-9-3p

By searching the miRDB and miRBase databases, we predicted that there was a possible binding site of miR-9-3p in the 3′-UTR of BLCAP mRNA, which may be a potential target of miR-9-3p (Fig. 4A). We then carried out a luciferase reporter analysis to clarify that prediction. The wild or mutant 3′-UTR of BLCAP mRNA was cloned into the luciferase vector (Fig. 4A). The luciferase activity of the BLCAP-wt reporter vector was significantly inhibited by the miR-9-3p mimic in TT cells compared to the control, while there was no obvious alteration in the luciferase activity of the BLCAP-mut reporter in TT cells transfected with miR-9-3p mimic (Fig. 4B). Additionally, the expression of BLCAP was significantly downregulated by the miR-9-3p mimic and upregulated by the miR-9-3p inhibitor in TT cells (Fig. 4C). These data indicate that BLCAP is a direct target of miR-9-3p in TT cells.

miR-9-3p Inhibits Apoptosis in TT Cells by Targeting BLCAP

Previous studies have reported that BLCAP plays a role in inducing S phase arrest and apoptosis in many human cancers[21,22]. To demonstrate whether BLCAP is involved in the regulation of miR-9-3p to TT cell apoptosis, we further employed adenovirus transduction and specific BLCAP siRNA to induce or inhibit BLCAP expression in TT cells. The efficiency of adenovirus transduction and BLCAP siRNA was confirmed by the RT-PCR assay. BLCAP expression was significantly decreased by the specific siRNA, while it was increased by the adenovirus in TT cells when compared with the control (Fig. 5A). Furthermore, the inhibited apoptosis of TT cells induced by the miR-9-3p mimic was enhanced by BLCAP overexpression (Fig. 5B). The levels of apoptosis were strongly decreased by BLCAP silencing in TT cells, which was not further influenced by the miR-9-3p inhibitor (Fig. 5C), implying that miR-9-3p inhibits apoptosis in TT cells by targeting BLCAP expression.

DISCUSSION

Dysregulation of miRNA expression has been reported in many pathological processes and has a key role in various biological processes, including cell proliferation, death, differentiation, and others[25]. As key endogenous regulators of gene expression, miRNAs have been considered to be implicated in the progression of multiple human diseases, including cancer[26]. In thyroid malignancies as well as MTC, dysregulation of miRNA expression has also been widely reported, including miR-146b, miR-38, miR-183, miR-199b, miR-323, miR-221 miR-127, miR-370, miR-21, miR-183, miR-154, miR-375, and miR-224[12,13]. These miRNAs have been recognized to be associated with carcinogenesis. In terms of miR-9-3p, previously named miR-9*, it has been found to be abnormally expressed and have a regulatory role in

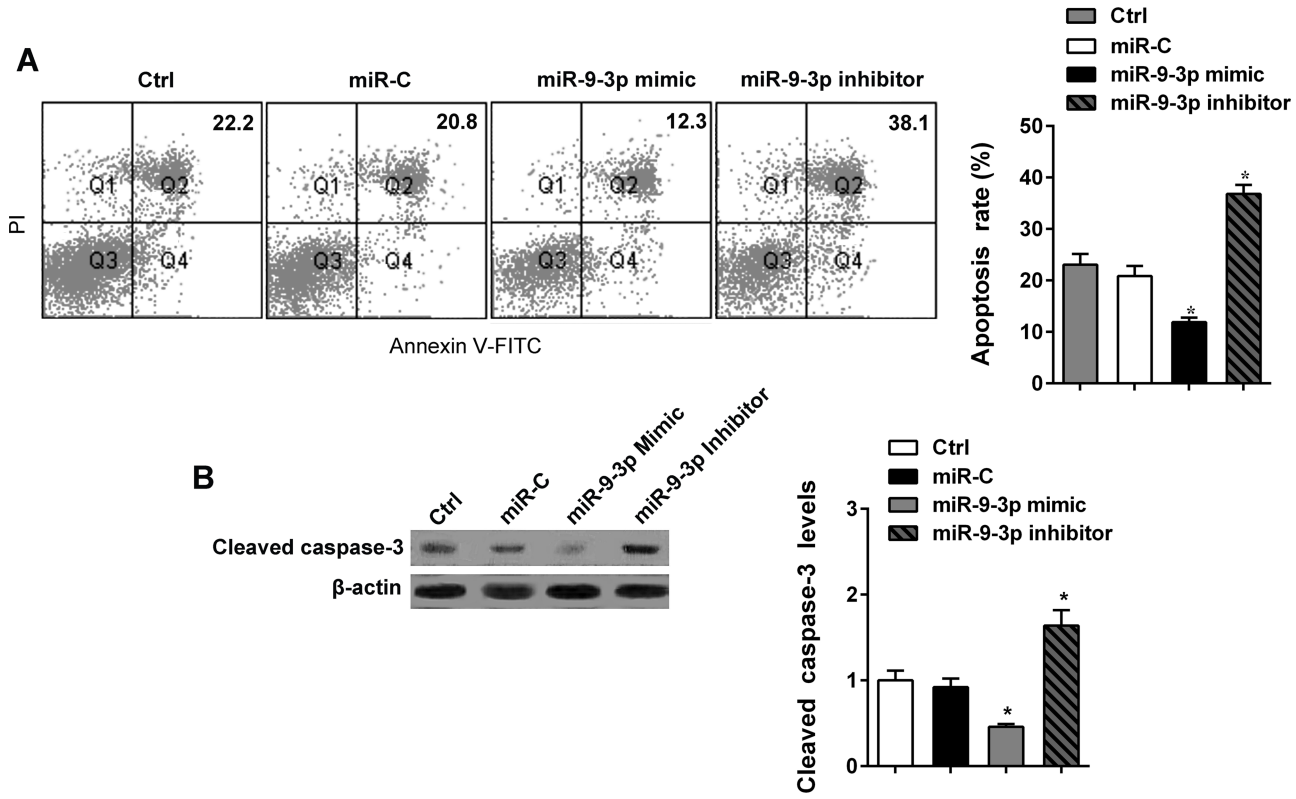

Figure 3. Effects of miR-9-3p on cell apoptosis in TT cells. TT cells were cultured (Ctrl), followed by transfection with the miR-9-3p mimic or inhibitor, as well as the negative control miRNA (miR-C). (A) Cell apoptosis was tested via flow cytometric analysis. (B) The levels of cleaved caspase 3 in cells were also evaluated by Western blot. $*p < 0.05$ versus Ctrl.

many diseases, such as trimethyltin-induced neurotoxicity, Huntington's disease, glioma, primary brain tumors, and others. Ogata et al. reported that miR-9* levels are elevated in the serum of rats treated by trimethyltin (TMT) and are considered to be associated with the evolution of neural cell death as a possible novel indicator of trimethyltin-induced neurotoxicity[18]. In another study, the reduction of bifunctional brain enriched miR-9/miR-9* was found to be correlated with Huntington's disease. miR-9/miR-9* could regulate the expression of REST and CoREST, two negative regulators in Huntington's disease[16]. Aberrant expression of miR-9-3p also exhibits an oncogenic function during tumorigenesis. Luo et al. reported that miR-9 expression is downregulated in gastric carcinoma in vitro and in vivo. Downregulated miR-9 could target the expression level of RAB34[27]. Additionally, the downregulation of miR-9-3p was proven to be involved in the regulation of chemoresistance and cancer stemness in glioma cells as well as differentiation in metastatic brain tumors[15,17].

A previous study reports that miR-9-3p is significantly upregulated in MTC[13]. Nevertheless, the role of miR-9-3p in the progression of MTC is still not completely clear. In the present study, we demonstrate the novel role of miR-9-3p in MTC. miR-9-3p expression is significantly upregulated in human MTC tumor tissues as well as cultured human MTC cell line TT cells. Further study found that the dysregulation of miR-9-3p could promote cell cycle progression and inhibit apoptosis in TT cells, indicating the role of miR-9-3p in human MTC tumorigenesis.

BLCAP, also known as bladder cancer 10 kDa protein (BC10), is a conserved transmembrane protein with 87 amino acids and two TMs[19]. The BLCAP gene was originally considered to be a novel tumor suppressor in human bladder carcinoma. It has been identified in multiple cancers, including HeLa cells, human Ewing's sarcoma, and tongue carcinoma. Zhao et al. reported that BLCAP exhibits a tumor suppressor function in cervical cancer. By targeting Rb1, BLCAP could arrest cell cycle progression and induce apoptosis in HeLa cells[21]. In another study, overexpression of BLCAP was found to inhibit cell growth and induce the apoptosis of human TC-135 Ewing's sarcoma cells in vitro by targeting expression of the apoptosis regulator B-cell lymphoma 2 (BCL-2) and the fusion transcription factor Ewing's sarcoma protein-

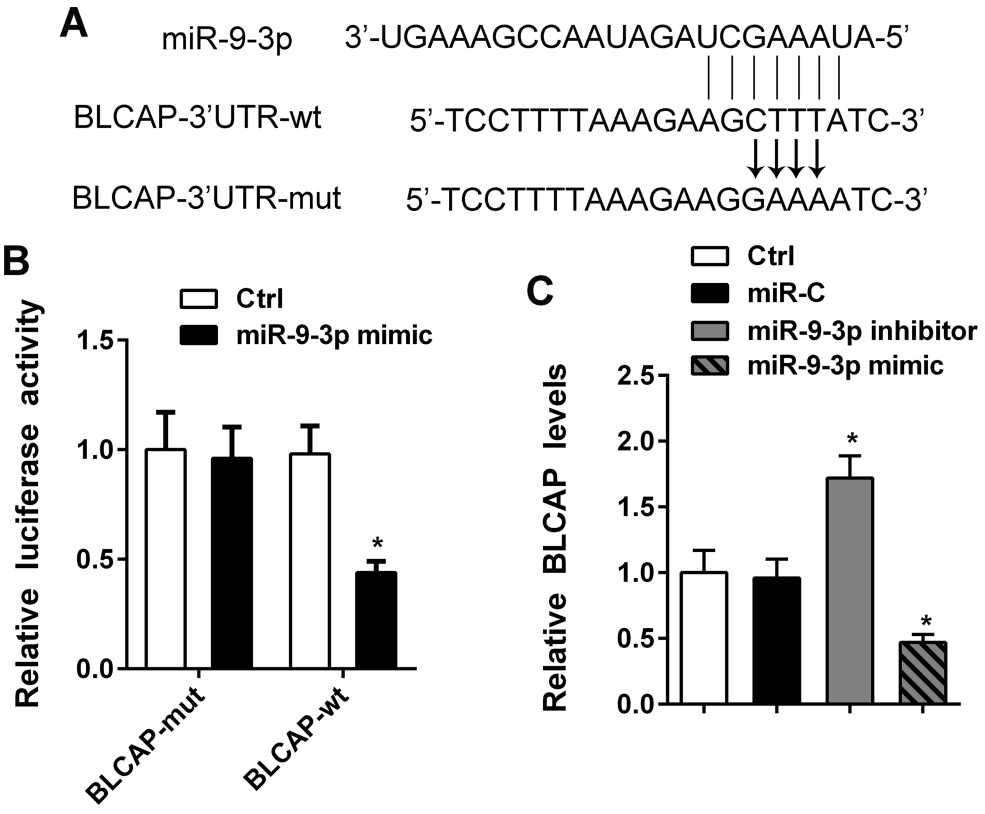

Figure 4. BLCAP is the direct target of miR-9-3p. (A) The prediction of the binding site of miR-9-3p in 3′-UTR of BLCAP. (B) Luciferase activity was tested in TT cells transfected with the miR-9-3p mimic as well as BLCAP-wt or BLCAP-mut. (C) RT-PCR was carried out to test the expression of BLCAP in TT cells. *$p<0.05$ versus Ctrl.

friend leukemia virus integration 1 (EWS-FLI1)[22]. In human tongue carcinoma, overexpressed BLCAP can also induce S phase arrest and apoptosis through p21(WAF1/CIP1) and the Bcl-XL/Bcl-2 pathway, which is independent of p53 and NF-κB signaling[20].

BLCAP is a highly conserved protein. There are a total of 17 editing sites in the human BLCAP pre-mRNA. It is reported that the editing events in three editing sites within the coding region of BLCAP can invoke change in the amino acid composition of proteins, contributing to the generation of eight different BLCAP protein isoforms[19]. In various cancers, not only is the expression of the BLCAP transcript dysregulated, but the editing events are also altered. Altered expression of BLCAP can inhibit cell survival and increase apoptosis in response to cytotoxic conditions. By searching the miRDB and miRBase databases and verification using luciferase reporter assay, we elucidated that BLCAP is a direct target of miR-9-3p in TT cells, with a binding site on the 3′-UTR.

Another approach we took to identify the role of miR-9-3p was to clarify the potential mechanisms by which miR-9-3p inhibits apoptosis. It is known that caspase 3 activity is essential for apoptosis. The BLCAP family of proteins plays a regulatory role in the regulation of caspase-dependent apoptosis via regulating p21(WAF1/CIP1) and the Bcl-XL/Bcl-2 pathway. By suppressing the release of caspase 3-dependent proteolytic cascade and mitochondrial cytochrome c, Bcl-2 can protect cells against apoptosis[28,29]. The shift in the Bax/Bcl-2 ratio may activate caspase 3 and initiate apoptosis[30]. We found that overexpression of miR-9-3p decreased the expression of caspase 3 in human TT cells, while the inhibited apoptosis rate of cells by miR-9-3p was reversed by BLCAP overexpression. The level of apoptosis was strongly decreased by BLCAP silencing in TT cells, which was not further influenced by the miR-9-3p inhibitor, implying that miR-9-3p inhibits apoptosis in TT cells by targeting the BLCAP expression.

In conclusion, upregulated miR-9-3p has a positive role in human MTC progression by regulating the growth and apoptosis of cancer cells via targeting BLCAP. This might represent a possible diagnosis or therapeutic target for MTC.

ACKNOWLEDGMENT: This research was funded by the Natural Science Foundation of Shaanxi Province (Grant No. 2016JM8118). The authors declare no conflicts of interest.

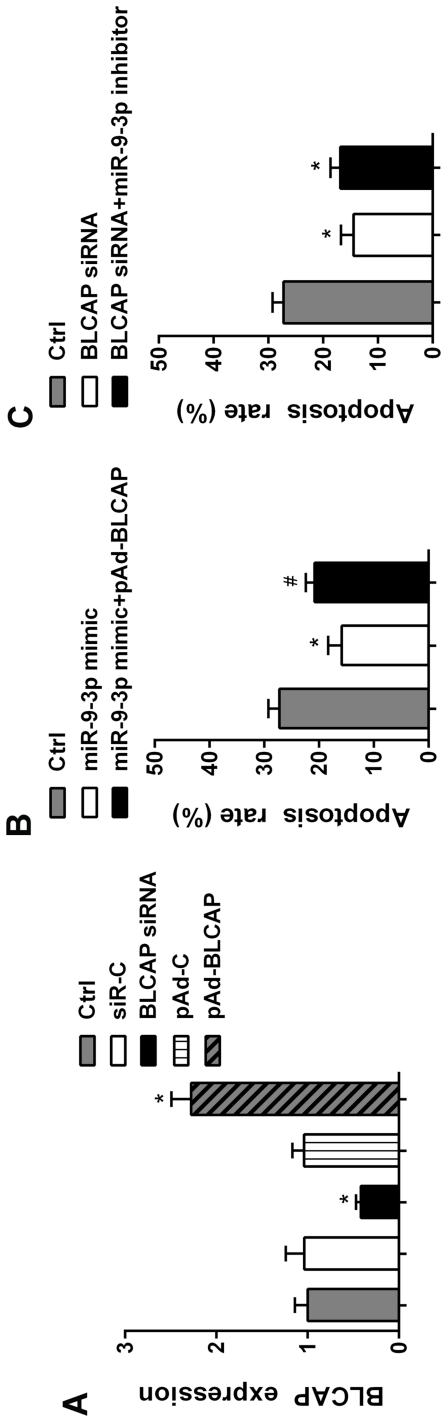

Figure 5. miR-9-3p inhibits apoptosis in TT cells by targeting BLCAP. (A) RT-PCR assay was performed to evaluate the efficiency of BLCAP siRNA and pAd-BLCAP in TT cells. (B) Apoptosis of TT cells was measured after transfection with pAd-BLCAP in the presence or absence of the miR-9-3p mimic. (C) Apoptosis was then tested in TT cells transfected with BLCAP siRNA in the presence or absence of the miR-9-3p inhibitor. *$p < 0.05$ versus Ctrl; #$p < 0.05$ versus miR-9-3p mimic.

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