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ORIGINAL RESEARCH

miR-218 overexpression suppresses tumorigenesis of papillary thyroid cancer via inactivation of PTEN/PI3K/AKT pathway by targeting Runx2

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Background: It was previously reported that downregulation of *miR-218* promoted thyroid cancer cell invasion, migration, and proliferation. However, the biological functions of *miR-218* and its possible regulatory mechanisms in papillary thyroid cancer (PTC) cells are still elusive.

Materials and methods: The expression levels of *miR-218* and Runx2 in PTC tissues and cells were determined by quantitative real-time PCR (qRT-PCR) and Western blot. The effects of *miR-218* overexpression on cell viability, invasion, apoptosis, and PTEN/PI3K/AKT pathway in PTC cells were evaluated by cell counting kit-8 assay, Transwell invasion assay, flow cytometry assay, and Western blot, respectively. Luciferase reporter assay and qRT-PCR were performed to identify the target of *miR-218*. Xenograft tumor experiment was performed to confirm the biological roles of *miR-218* and its potential mechanisms in vivo.

Results: *miR-218* expression was downregulated and Runx2 expression was upregulated in PTC tissues and cells. Overexpression of *miR-218* suppressed viability and invasion, and induced apoptosis of PTC cells in vitro, while Runx2 overexpression greatly abolished these effects. *miR-218* overexpression inactivated the PTEN/PI3K/AKT pathway, which was abated by Runx2 upregulation. Additionally, Runx2 was validated to be a direct target of *miR-218*. Moreover, enforced expression of *miR-218* inhibited tumor growth and Runx2 expression, and blocked PTEN/PI3K/AKT pathway in vivo.

Conclusion: *miR-218* overexpression suppresses the tumorigenesis of PTC via downregulating PTEN/PI3K/AKT pathway by targeting Runx2, which indicates that *miR-218* may be a potential therapeutic target for human PTC.

Keywords: miR-218, papillary thyroid cancer, PTEN/PI3K/AKT, Runx2

Introduction

Thyroid cancer (TC) is the most prevalent malignancy of endocrine organs with a rapidly increasing incidence in female in the past three decades.¹ Almost 64,300 new cases of TC were diagnosed in 2016, and it has been estimated that TC results in nearly 1,980 cancer-associated mortalities per year.² Papillary thyroid cancer (PTC) is the most common histopathological subtype of TC, accounting for approximately 90% of all TC cases.³ Despite the advances in radiotherapy and surgical treatment, 10%–15% of PTC patients are prone to relapse and develop distant metastases, indicative of poor response to standard therapy and an unfavorable clinical outcome.³ Thus, the molecular mechanisms underlying the tumorigenesis and progression of PTC are worthy of further investigation to develop a more effective therapeutic strategy.

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miRNAs, a class of small endogenous noncoding RNAs (18-22 nucleotides), can post-transcriptionally modulate gene expressions by pairing with the complementary sequences at 3'UTR of target gene mRNAs, leading to mRNA degradation and/or translational blockage.4 miRNAs have been considered as a crucial regulator of various biological and pathological processes, such as cell proliferation, migration, invasion, apoptosis, and tumorigenesis.⁵ miRNAs are identified as either oncogenes or tumor suppressors in different types of cancers.⁶ Additionally, several studies reveal that dysregulation of miRNAs, such as miR-199a-3p, miR-21, and miR-217, is associated with TC pathogenesis and progression.7-9 miR-218, an miRNA transcribed from two loci located on chromosome 4p15.31 (miR-218-1) and 5q35.1 (miR-218-2), is found to be downregulated in several cancers and associated with cell invasiveness.^{10,11} Many documents also demonstrate the anticancer functions of miR-218.12,13 A previous study reported that downregulation of miR-218-2 and its host gene SLIT3 promotes TC cell invasion, migration, and proliferation.¹⁴ However, the regulatory mechanisms by which miR-218 exerts its biological functions in TC cells are still unclear.

Runx2, a member of Runx family, is a determinant regulator of differentiation of osteoblasts and formation of bone.¹⁵ Aberrant expression of Runx2 is implicated in proliferation, apoptosis, and pathogenesis of several tumor cells.¹⁶ Moreover, the oncogenic role of Runx2 in migration and invasion has been well documented in certain types of tumors.^{17,18} Notably, a previous study showed that *miR-218* could directly target Runx2 to regulate cisplatin chemosensitivity of non-small-cell lung cancer.¹⁹ However, whether *miR-218* elicits its biological effects by modulating Runx2 in TC cells is undefined.

PTEN, the upstream molecule of PI3K/AKT pathway, is a tumor suppressor that participates in cell growth, apoptosis, invasion, and migration.²⁰ It has been generally believed that the PTEN/PI3K/AKT signaling pathway plays an important role in cancer development and thus is an attractive target for molecular therapy.²¹ In this study, the biological roles of *miR-218* in PTC tumorigenesis were explored in vitro and in vivo.

Materials and methods Patients and clinical tissue specimens

A total of 21 pairs of primary PTC tissues and matched adjacent normal tissues were obtained from PTC patients confirmed by pathological analysis at Chinese People's Liberation Army General Hospital (Beijing, China). Specimens were immediately snap-frozen in liquid nitrogen following surgery until RNA extraction. This study was approved by the Ethics Committee of Chinese People's Liberation Army General Hospital, and written informed consents were signed by all participants.

Cell lines and cultures

Human PTC cell lines (TPC-1 and K-1) and human thyroid epithelial cell line Nthy-ori3-1 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were maintained in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (HyClone, Logan, UT, USA) and 100 U/mL penicillin/ streptomycin (Sigma-Aldrich, St Louis, MO, USA) at 37°C in a humidified incubator with 5% CO₂.

Cell transfection

miR-218 mimics (*miR-218-5p*), *miR-218* inhibitor, control miRNA (miR-control), pcDNA-Runx2, pcDNA empty vector (vector), siRNA against Runx2 (si-Runx2), and siRNA control (si-control) were all obtained from GenePharma Co., Ltd. (Shanghai, China). TPC-1 and K-1 cells at approximately 80% confluence were transfected with mimics, siRNAs, or plasmids using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). At 48 hours post-transfection, cells were harvested and subjected to next analyses.

Quantitative real-time PCR

Total RNA from tissues and cultured cells was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. For miR-218 expression analysis, miRNAs were reversely transcribed using the TaqMan MiRNA Reverse Transcript Kit (Thermo Fisher Scientific, Waltham, MA, USA). To determine the expression of Runx2 mRNA, total RNA was inversely transcribed to cDNA using Prime Script 1st Strand cDNA Synthesis Kit (TaKaRa, Kusatsu, Japan). Real-time PCR was performed by using SYBR Premix Ex Taq[™] (TaKaRa) on an Applied Biosystems 7500 Sequence Detection System (Thermo Fisher Scientific). Relative expressions of miR-218 and Runx2 mRNA were determined using the $2^{-\Delta\Delta Ct}$ method, with U6 small nuclear or GAPDH as a normalization control. Primer sequences used for quantitative real-time PCR (qRT-PCR) were as follows: Runx2 forward, 5'-CCGCCTCAGTGATTTAGGGC-3' and reverse, 5'-GGGTCTGTAATCTGACTCTGTCC-3'; miR-218 (miR-218-5p)forward,5'-TTGTGCTTGATCTAACCATGT-3' and reverse, 5'-CAGTGCGTGTCGTGGAGT-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'; GAPDH forward, 5'-TATGATGATATCAAGAG GGTAGT-3' and reverse, 5'-TGTATCCAAACTCATTG TCATAC-3'.

Western blot assay

Tumor samples or cultured cells were washed three times with PBS and lysed in RIPA lysis buffer (Beyotime Biotechnology, Haimen, China) containing 1% protease inhibitors (Pierce, Rockford, IL, USA) for 30 minutes on ice. The protein concentration was quantified using a BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of protein samples (30 µg) were separated on 10% SDS-PAGE, and then transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). After blocking with 5% nonfat milk, the membrane was incubated with primary antibodies against Runx2 (1:1,000), total AKT (t-AKT) (1:1,000), phosphorylated AKT (p-AKT) (1:500), PTEN (1:1,000), E-cadherin (1:1,000), N-cadherin (1:1,000), vimentin (1:1,000), mTOR (1:1,000), GSK-3β (1:500), or β-actin (1:2,000) (Abcam Inc., Cambridge, UK) overnight at 4°C. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich) for 2 hours at room temperature, the blots were visualized using an enhanced chemiluminescence kit (Santa Cruz, Dallas, TX, USA).

Cell viability assay

The cell viability was evaluated using cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Briefly, the cells were maintained in 96-well plates at a density of 5,000 cells per well and transfected with indicated oligonucleotides or plasmids. At 48 hours post-transfection, the cells were incubated in DMEM containing $10 \,\mu$ L of CCK-8 solution at 37°C for 2 hours. The absorbance at 450 nm of each well was determined using a microplate reader (Molecular Devices, San Jose, CA, USA).

Flow cytometry analysis of apoptosis

The transfected cells were harvested by digestion with trypsinization and washed twice with cold PBS. Then, cells were stained with Annexin V-FITC Apoptosis Detection Kit I (BD, Franklin Lakes, NJ, USA) as previously reported.²² Apoptotic cells were identified and analyzed using an FACScan flow cytometer (FACS Calibur; BD).

Transwell invasion assay

Cell invasion was examined using Matrigel Invasion Chambers (BD Biosciences, San Jose, CA, USA) with inserts containing an 8 μ m pore-size membrane with Matrigel (BD Biosciences). The transfected cells (1×10⁵) were seeded into the upper chamber with serum-free medium, and the lower chamber was filled with 600 μ L DMEM with 10% FBS as a chemoattractant. After incubation for 24 hours, non-invading cells were scraped off from the top well. Cells that invaded to the lower surface of the chambers were fixed with methanol, stained with 0.1% crystal violet (Beyotime Biotechnology) for 5 minutes, and counted in five visual fields randomly under an inverted microscope.

Luciferase reporter assay

The fragments of Runx2 mRNA 3'UTR containing the binding sites of *miR-218* were amplified and cloned into the downstream of a luciferase reporter gene in the pmirGLO vector (Promega, Madison, WI, USA), namely pmirGLO-Runx2-3'UTR-WT. The mutant construct (pmirGLO-Runx2-3'UTR-MUT) that carried the mutant Runx2 3'UTR region was generated using the QuikChange[®] site-directed mutagenesis kit (Stratagene, San Diego, CA, USA). Cells were cotransfected with 100 ng luciferase constructs, 20 ng Renilla luciferase control vector (pRL-TK), and 50 nM *miR-218* or miR-control using Lipofectamine 2000 (Thermo Fisher Scientific). After 48 hours, the luciferase activity was detected using the dual-luciferase reporter assay system (Promega) and normalized to Renilla luciferase activity.

Xenograft tumor experiment

The animal procedures were conducted according to the protocols approved by the Institutional Animal Care and Use Committee of Chinese People's Liberation Army General Hospital. The whole experiment process was also carried out with approval of the Research Ethic Committee of Chinese People's Liberation Army General Hospital. Four-weekold male BALB/c athymic nude mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) and maintained under specific pathogenfree conditions. TPC-1 cells (5×10^6) transfected with lentiviral vectors encoding miR-218 or miR-control were subcutaneously injected to the right flank region of nude mice (six mice per group). When the tumor was visible, tumor volume was monitored every 5 days with vernier calipers using the formula: volume (mm³) = length/2 × width². Thirtyfive days later, mice were sacrificed, and the tumor masses were removed and weighed. The resected tumor tissues were collected for protein and total RNA extraction.

Statistical analysis

Experimental data were represented as mean \pm SD and analyzed using SPSS 19.0 software (IBM, Armonk, NY, USA). Statistical comparisons were analyzed using Student's *t*-test and one-way ANOVA. Data were considered statistically significant at *P*-value less than 0.05.

Results

miR-218 was downregulated and Runx2 was upregulated in PTC tissues

The expressions of *miR-218* and Runx2 in PTC tissues and matched adjacent normal tissues were examined by qRT-PCR. As shown in Figure 1A and B, *miR-218* expression was conspicuously reduced and Runx2 mRNA level was strikingly elevated in PTC tissues compared to adjacent noncancerous tissues. Interestingly, an inverse correlation between *miR-218* and Runx2 mRNA expression was also observed in PTC tissues (Figure 1C).

miR-218 was downregulated and Runx2 was upregulated in PTC cells

The expression levels of *miR-218* and Runx2 in PTC cells were further confirmed by qRT-PCR and Western blot.

miR-218 expression was significantly lower in PTC cell lines (TPC-1 and K-1) than human thyroid epithelial cell line Nthy-ori3-1 (Figure 2A). Runx2 expression was remarkably higher at both mRNA and protein levels in TPC-1 and K-1 cells than Nthy-ori3-1 cells (Figure 2B and C). These results further indicated that *miR-218* and Runx2 may be implicated in the development of PTC.

miR-218 overexpression suppressed cell viability, invasion, and induced apoptosis of PTC cells in vitro

To confirm the biological roles of *miR-218* in PTC progression, K-1 and TPC-1 cells were transiently transfected with *miR-218* mimic or miR-control. Subsequently, qRT-PCR, CCK-8, Transwell invasion assay, and flow cytometry analysis were performed at different time points. As illustrated in Figure 3A, a significant increase of *miR-218* expression was observed in K-1 and TPC-1 cells following transfection of mi-218 mimic. The results of CCK-8 assay disclosed that ectopic expression of *miR-218* led to a marked decrease of cell viability in both K-1 and TPC-1 cells compared with miR-control-transfected cells (Figure 3B). As compared with miR-control group, the invasive abilities were dramatically repressed in K-1 (Figure 3C) and TPC-1 (Figure 3D) cells after treatment with *miR-218* mimic. Flow cytometry analysis indicated that *miR-218* overexpression



Figure I Expressions of miR-218 and Runx2 in PTC tissues.

Notes: qRT-PCR was performed to detect the expressions of (A) miR-218 and (B) Runx2 mRNA in PTC tissues and matched adjacent normal tissues. (C) Correlation analysis between miR-218 and Runx2 mRNA expressions in PTC tissues.

Abbreviations: PTC, papillary thyroid cancer; qRT-PCR, quantitative real-time PCR.



Figure 2 Expressions of miR-218 and Runx2 in PTC cells.

Notes: (A) The expressions of *miR*-218 in PTC cell lines (TPC-1 and K-1) and human thyroid epithelial cell line Nthy-ori3-1 were evaluated by qRT-PCR. The expression levels of Runx2 at (B) mRNA and (C) protein levels in TPC-1, K-1, and Nthy-ori3-1 cells were assessed by qRT-PCR and Western blot. *P<0.05. Abbreviations: PTC, papillary thyroid cancer; qRT-PCR, quantitative real-time PCR.

remarkably increased the apoptotic rates of K-1 (Figure 3E) and TPC-1 (Figure 3F) cells with respect to the control group. Collectively, these data demonstrated that *miR-218* acted as a tumor suppressor in the development of PTC by inhibiting cell viability and invasion and inducing apoptosis in vitro.

miR-218 upregulation repressed epithelial–mesenchymal transition (EMT) of PTC cells

Accumulating evidence has demonstrated that EMT is a crucial biological process for metastasis and invasion of tumor cells derived from epithelial cells, accompanied by the decrease of E-cadherin and the increase of mesenchymal markers (N-cadherin and vimentin).²³ The expressions of EMT markers were determined to investigate the effect of *miR-218* on EMT in PTC cells. Western blot analysis showed that the protein level of E-cadherin was significantly enhanced, while N-cadherin and vimentin levels were dramatically reduced in *miR-218* mimic-transfected TPC-1 cells relative to miR-controltransfected cells (Figure 4). In all, *miR-218* overexpression suppressed EMT of PTC cells.

miR-218 directly targeted Runx2 and regulated its expression in PTC cells

Since a previous study reported that *miR-218* could directly target Runx2 in non-small-cell lung cancer,¹⁹ the same mechanism was further explored in PTC cells. As shown in Figure 5A, a complementary sequence of *miR-218* was presented within the 3'UTR of Runx2. Then, luciferase reporter vectors containing the wild-type or mutantbinding sites of *miR-218* in Runx2 were constructed and cotransfected with *miR-218* or miR-control into K-1 and TPC-1 cells. The results showed that cotransfection with *miR-218* and wild-type Runx2 3'UTR dramatically inhibited luciferase activity, while no obvious differences were observed in luciferase expression of pmirGLO-Runx2-3'UTR-MUT between *miR-218* and miR-control group in both K-1 and TPC-1 cells (Figure 5B). To confirm the effect of *miR-218* on Runx2 expression, K-1 and TPC-1 cells were transfected with *miR-218* mimic, *miR-218* inhibitor, or miR-control. qRT-PCR analysis revealed that *miR-218* transfection substantially reduced the expression

of Runx2, while *miR-218* inhibitor markedly improved the expression of Runx2 in K-1 and TPC-1 cells (Figure 5C). Taken together, these data suggested that Runx2 was a direct target of *miR-218*.

TPC-1

TPC-1

miR-218

miR-218

miR-218



Figure 3 (Continued)

miR-218



Figure 3 Effects of miR-218 overexpression on tumorigenesis of PTC in vitro.

Notes: K-I and TPC-I cells were transfected with miR-218 or miR-control, and further experiments were performed at 24 or 48 hours post-transfection. (A) qRT-PCR analysis of miR-218 expression in transfected K-I and TPC-I cells. (B) CCK-8 assay was conducted in transfected K-I and TPC-I cells to measure the cell viability. Cell invasive ability was examined by Transwell invasion assay in transfected (C) K-I and (D) TPC-I cells (magnification \times 200). Flow cytometry analysis of apoptosis in transfected (E) K-I and (F) TPC-I cells. *P<0.05.

Abbreviations: CCK-8, cell counting kit-8; FITC, fluorescein isothiocyanate; PI, propidium iodide; PTC, papillary thyroid cancer; qRT-PCR, quantitative real-time PCR.

miR-218 suppressed cell viability and invasion, and induced apoptosis of PTC cells via inactivating PTEN/PI3K/AKT pathway by targeting Runx2 in vitro

To further clarify whether the inhibitory effect of *miR-218* on tumorigenesis of PTC was mediated by Runx2, rescue

experiments were performed by transfecting *miR-218* alone or with pcDNA-Runx2 into TPC-1 cells. CCK-8 assay showed that Runx2 overexpression significantly reversed *miR-218*-mediated viability suppression in TPC-1 cells (Figure 6A). Meanwhile, enforced expression of *miR-218* markedly reduced the invasive ability of TPC-1



Figure 4 miR-218 inhibits EMT of PTC cells.

Notes: Western blot was conducted to determine the expression levels of E-cadherin, N-cadherin, and vimentin in TPC-1 cells transfected with miR-control or miR-218 mimic. *P<0.05.

Abbreviations: EMT, epithelial-mesenchymal transition; PTC, papillary thyroid cancer.



Figure 5 miR-218 directly targets Runx2 and regulates its expression in PTC cells.

Notes: (A) Diagram of the binding sites between miR-218 and Runx2 3'UTR. (B) The relative luciferase activity was measured in K-1 and TPC-1 cells after transfection with miR-218 or miR-control and luciferase reporter vectors with wild-type (WT) or mutant (MUT) Runx2 3'UTR. (C) Western blot analysis of Runx2 expression level in K-1 and TPC-1 cells transfected with miR-218 mimic, miR-218 inhibitor, or miR-control. *P<0.05.

Abbreviations: NC, normal control; PTC, papillary thyroid cancer.

cells, whereas transfection of pcDNA-Runx2 obviously abolished this effect, as determined by Transwell invasion assay (Figure 6B). Flow cytometry analysis uncovered that miR-218-induced apoptosis of TPC-1 cells was remarkably attenuated by Runx2 upregulation (Figure 6C). Since PTEN/ PI3K/AKT pathway is proved as an important regulator of various biological processes in several tumors including TC,²⁴ the effect of miR-218 and Runx2 on the PTEN/PI3K/ AKT pathway was examined. The expression levels of p-AKT (Ser473), t-AKT, and PTEN were detected in TPC-1 cells transfected with si-Runx2, miR-218, miR-218+ pcDNA-Runx2, or matched controls by Western blot. As shown in Figure 6D and E, Runx2 knockdown or miR-218 overexpression effectively decreased the levels of p-AKT (Ser473) and promoted PTEN expression. Moreover, overexpression of Runx2 significantly abrogated the inhibitory effects of miR-218 on PTEN/PI3K/AKT pathway, suggesting that miR-218 overexpression blocked the PTEN/PI3K/AKT pathway by regulating Runx2. Taken together, these findings revealed that *miR-218* exerted tumor-suppressive effect in PTC cells via inactivation of PTEN/PI3K/AKT pathway by modulating Runx2 in vitro.

miR-218 overexpression inhibited tumor growth in PTC partly through Runx2/ PTEN/PI3K/AKT pathway in vivo

To explore the role of *miR-218* in tumorigenesis of PTC in vivo, TPC-1 cells transfected with *miR-218* or miRcontrol were injected into nude mice. Then, the mice body weight was monitored every 5 days and it was found that *miR-218* overexpression had no obvious effect on the body weight of mice (Figure 7A). As presented by growth curves of tumors in Figure 7B, *miR-218* upregulation significantly lowered tumor volumes compared to miR-control group. Also, a marked reduction of tumor weight was observed in *miR-218*-overexpressing group compared with miR-control group (Figure 7C). In addition, Runx2 expression at mRNA and protein levels in resected tumors was inhibited in



Figure 6 miR-218 exerts anticancer effects in PTC via inactivation of PTEN/PI3K/AKT pathway by targeting Runx2 in vitro. Notes: After TPC-1 cells were transfected with miR-218 alone or in combination with pcDNA-Runx2, cell viability, invasion, and apoptosis were examined by (**A**) CCK-8 assay, (**B**) Transwell invasion assay, and (**C**) flow cytometry analysis, respectively. (**D** and **E**) The protein levels of phosphorylated AKT (p-AKT) (Ser473), total AKT (t-AKT), and PTEN were determined by Western blot in TPC-1 cells transfected with si-Runx2, miR-218, miR-218+ pcDNA-Runx2, or respective controls blot. *P<0.05. **Abbreviations:** CCK-8, cell counting kit-8; PTC, papillary thyroid cancer.



Figure 7 miR-218 overexpression inhibits PTC tumor growth in vivo.

Notes: TPC-1 cells transfected with lentiviral vectors containing *miR*-218 or miR-control were injected into nude mice. The mice (**A**) body weights and (**B**) tumor volumes were monitored every 5 days. (**C**) The mice were killed after 35 days, and the tumor weights were measured. The expression of Runx2 at mRNA and protein levels in removed tumors was assessed by (**D**) qRT-PCR and (**E**) Western blot. (**F**) The protein levels of phosphorylated AKT (p-AKT) (Ser473), PTEN, and total AKT (t-AKT) in excised tumors were determined by Western blot. (**G**) The protein levels of p-mTOR, t-mTOR, p-GSK-3 β , and t-GSK-3 β in excised tumors were examined by Western blot. **P*<0.05.

Abbreviations: PTC, papillary thyroid cancer; qRT-PCR, quantitative real-time PCR.

miR-218-overexpressing group than miR-control group (Figure 7D and E). Furthermore, transfection of *miR-218* resulted in an obvious decrease in p-AKT (Ser473) expression and a prominent increase in PTEN expression, as compared to the control group (Figure 7F). Meanwhile, the expression levels of the downstream targets of PTEN/PI3K/ AKT pathway including mTOR and GSK-3 β were evaluated by Western blot. The results revealed that ectopic expression of *miR-218* evidently limited the phosphorylation of mTOR (p-mTOR) and GSK-3 β (p-GSK-3 β) (Figure 7G). All these data confirmed the tumor-suppressive function of *miR-218* in PTC via Runx2/PTEN/PI3K/AKT in vivo.

Discussion

The oncogenic or tumor-suppressive roles of miRNAs have been well-documented in numerous human tumors, and great efforts have been made to better understand their regulatory mechanism. Accumulating evidence has indicated that miRNAs play essential roles in tumorigenesis of human TC due to their dysregulation.^{25–28} In this study, we found that miR-218 expression was downregulated in PTC tissue specimens and cell lines, in accordance with a previous study.¹⁴ Functional analyses revealed that exogenous upregulation of miR-218 suppressed cell viability, invasion, and EMT, promoted apoptosis of PTC cells in vitro, and inhibited tumor growth in vivo, suggesting that miR-218 acted as a tumor suppressor in the development of PTC. The anticancer role of miR-218 was also elucidated in other types of tumors. For example, miR-218 overexpression was reported to inhibit proliferation and invasion of gastric cancer cells by regulating the expression of angiopoietin-2.29 miR-218 inhibited tumor growth and increased chemosensitivity to cisplatin in prostate cancer via negatively regulating BCAT1 protein expression.³⁰ In glioma cells, miR-218 affected cell proliferation, apoptosis, and invasion by negatively modulating HMGB-mediated suppression of receptor for advanced glycation end products.³¹

Runx2, a runt-related transcription factor, is a crucial regulator of normal bone development, homeostasis, and remodeling.³² Upregulation of Runx2 expression has been largely reported in various cancers, such as prostate cancer, breast cancer, and TC.³³ It is well documented that Runx2 is involved in tumor formation, progression, and metastasis. For instance, Runx2 promoted the invasion and metastasis of human gastric cancer cells in vitro and in vivo by transcriptionally upregulating the chemokine receptor *CXCR4*.³⁴ The strong expression of Runx2 regulates proliferation, migration, and invasiveness of PTC cells by activating a panel of genes involved in matrix degradation and cellular invasion.³⁵

miRNAs, such as *miR-446*,³⁶ *miR-221*,³⁷ and *miR-203*,³⁸ and these miRNAs negatively regulated its expression and activity in different physiological or pathological conditions. In the present study, Runx2 expression was demonstrated to be upregulated in PTC tissues and cells, and negatively correlated with *miR-218* expression in PTC tissues. Moreover, *miR-218* could direct target Runx2 to inhibit its expression. More interestingly, Runx2 overexpression significantly reversed the effects of *miR-218* on viability, invasion, and apoptosis, indicating that Runx2 may partly mediate the biological functions of *miR-218* in PTC cells.

PTEN/PI3K/AKT pathway plays a central role in multiple cellular processes, such as cell proliferation, cell cycle, and survival, in different human cancers including TC.^{39,40} Additionally, a recent study revealed that Runx2 was important for the activation of the PI3K/AKT signaling pathway,⁴¹ and Runx2 downregulation repressed the activation of PI3K/AKT signaling pathway.^{41,42} Our study revealed that *miR-218* upregulation reduced p-AKT expression (Ser4473) and elevated PTEN level, while Runx2 overexpression markedly abated these effects, indicating that *miR-218* blocked the PTEN/PI3K/AKT pathway by targeting Runx2. Based on all these results, a novel molecular mechanism of *miR-218* in regulation of PTC tumorigenesis was proposed (Figure 8).



Figure 8 A schematic model of the mechanism of *miR-218* in PTC. **Abbreviation:** PTC, papillary thyroid cancer.

Conclusion

We first confirmed that *miR-218* was downregulated and Runx2 was upregulated in human PTC tissues and cells. Furthermore, ectopic expression of *miR-218* suppressed tumorigenesis of PTC via inactivation of PTEN/PI3K/ AKT pathway by targeting Runx2. Our study elucidated a novel molecular basis of *miR-218* in PTC development and provided a promising therapeutic target for PTC.

Disclosure

The authors report no conflicts of interest in this work.

References

- Viola D, Valerio L, Molinaro E, et al. Treatment of advanced thyroid cancer with targeted therapies: ten years of experience. *Endocr Relat Cancer*. 2016;23(4):R185–R205.
- Siegel RL, Miller KD, Jemal A, Statistics C. Cancer statistics, 2016. CA Cancer J Clin. 2016;66(1):7–30.
- 3. Ito Y, Jikuzono T, Higashiyama T, et al. Clinical significance of lymph node metastasis of thyroid papillary carcinoma located in one lobe. *World J Surg.* 2006;30(10):1821–1828.
- 4. Esquela-Kerscher A, Slack FJ. Oncomirs microRNAs with a role in cancer. *Nat Rev Cancer*. 2006;6(4):259–269.
- Muhammad N, Bhattacharya S, Steele R, Ray RB. Anti-miR-203 suppresses ER-positive breast cancer growth and stemness by targeting SOCS3. *Oncotarget*. 2016;7(36):58595–58605.
- Song HM, Luo Y, Li DF, et al. MicroRNA-96 plays an oncogenic role by targeting FOXO1 and regulating AKT/FOXO1/Bim pathway in papillary thyroid carcinoma cells. *Int J Clin Exp Pathol.* 2015;8(9): 9889–9900.
- Minna E, Romeo P, de Cecco L, et al. miR-199a-3p displays tumor suppressor functions in papillary thyroid carcinoma. *Oncotarget*. 2014;5(9):2513–2528.
- Zhang J, Yang Y, Liu Y, et al. MicroRNA-21 regulates biological behaviors in papillary thyroid carcinoma by targeting programmed cell death 4. *J Surg Res.* 2014;189(1):68–74.
- Lin Y, Cheng K, Wang T, et al. miR-217 inhibits proliferation, migration, and invasion via targeting AKT3 in thyroid cancer. *Biomed Pharmacother*. 2017;95:1718–1724.
- Yu J, Wang Y, Dong R, Huang X, Ding S, Qiu H. Circulating microRNA-218 was reduced in cervical cancer and correlated with tumor invasion. J Cancer Res Clin Oncol. 2012;138(4):671–674.
- Liu Y, Yan W, Zhang W, et al. MiR-218 reverses high invasiveness of glioblastoma cells by targeting the oncogenic transcription factor LEF1. Oncol Rep. 2012;28(3):1013–1021.
- Uesugi A, Kozaki K, Tsuruta T, et al. The tumor suppressive microRNA miR-218 targets the mTOR component Rictor and inhibits AKT phosphorylation in oral cancer. *Cancer Res.* 2011;71(17):5765–5778.
- Venkataraman S, Birks DK, Balakrishnan I, et al. MicroRNA 218 acts as a tumor suppressor by targeting multiple cancer phenotype-associated genes in medulloblastoma. *J Biol Chem.* 2013;288(3):1918–1928.
- Guan H, Wei G, Wu J, et al. Down-regulation of miR-218-2 and its host gene SLIT3 cooperate to promote invasion and progression of thyroid cancer. *J Clin Endocrinol Metab.* 2013;98(8):E1334–E1344.
- Chen H, Ghori-Javed FY, Rashid H, et al. Runx2 regulates endochondral ossification through control of chondrocyte proliferation and differentiation. *J Bone Miner Res.* 2014;29(12):2653–2665.
- Eliseev RA, Dong YF, Sampson E, et al. Runx2-mediated activation of the Bax gene increases osteosarcoma cell sensitivity to apoptosis. *Oncogene*. 2008;27(25):3605–3614.
- Cohen-Solal KA, Boregowda RK, Lasfar A. RUNX2 and the PI3K/ AKT axis reciprocal activation as a driving force for tumor progression. *Mol Cancer*. 2015;14(1):137.

- Pratap J, Wixted JJ, Gaur T, et al. Runx2 transcriptional activation of Indian Hedgehog and a downstream bone metastatic pathway in breast cancer cells. *Cancer Res.* 2008;68(19):7795–7802.
- Xie J, Yu F, Li D, Zhu X, Zhang X, Lv Z. MicroRNA-218 regulates cisplatin (DPP) chemosensitivity in non-small cell lung cancer by targeting RUNX2. *Tumour Biol.* 2016;37(1):1197–1204.
- Miao Y, Zheng W, Li N, et al. MicroRNA-130b targets PTEN to mediate drug resistance and proliferation of breast cancer cells via the PI3K/ Akt signaling pathway. *Sci Rep.* 2017;7:41942.
- Capodanno A, Camerini A, Orlandini C, et al. Dysregulated PI3K/Akt/ PTEN pathway is a marker of a short disease-free survival in nodenegative breast carcinoma. *Hum Pathol.* 2009;40(10):1408–1417.
- Yi Y, Chen J, Jiao C, et al. Upregulated miR-193a-3p as an oncogene in esophageal squamous cell carcinoma regulating cellular proliferation, migration and apoptosis. *Oncol Lett.* 2016;12(6):4779–4784.
- Tania M, Khan MA, Fu J. Epithelial to mesenchymal transition inducing transcription factors and metastatic cancer. *Tumour Biol.* 2014; 35(8):7335–7342.
- Xu J, Cai J, Jin X, et al. PIG3 plays an oncogenic role in papillary thyroid cancer by activating the PI3K/AKT/PTEN pathway. *Oncol Rep.* 2015;34(3):1424–1430.
- He H, Jazdzewski K, Li W, et al. The role of microRNA genes in papillary thyroid carcinoma. *Proc Natl Acad Sci U S A*. 2005;102(52): 19075–19080.
- Swierniak M, Wojcicka A, Czetwertynska M, et al. In-depth characterization of the microRNA transcriptome in normal thyroid and papillary thyroid carcinoma. *J Clin Endocrinol Metab.* 2013;98(8): E1401–E1409.
- Rosignolo F, Memeo L, Monzani F, et al. MicroRNA-based molecular classification of papillary thyroid carcinoma. *Int J Oncol.* 2017;50(5): 1767–1777.
- Pishkari S, Paryan M, Hashemi M, Baldini E, Mohammadi-Yeganeh S. The role of microRNAs in different types of thyroid carcinoma: a comprehensive analysis to find new miRNA supplementary therapies. *J Endocrinol Invest*. 2018;41(3):269–283.
- Tang S, Wang D, Zhang Q, Li L. miR-218 suppresses gastric cancer cell proliferation and invasion via regulation of angiopoietin-2. *Exp Ther Med.* 2016;12(6):3837–3842.
- Zhu W, Shao Y, Peng Y. MicroRNA-218 inhibits tumor growth and increases chemosensitivity to CDDP treatment by targeting BCAT1 in prostate cancer. *Mol Carcinog.* 2017;56(6):1570–1577.
- Gu J, Xu R, Li Y, Zhang J, Wang S. MicroRNA-218 modulates activities of glioma cells by targeting HMGB1. *Am J Transl Res.* 2016; 8(9):3780–3790.
- 32. Komori T. The functions of Runx family transcription factors and Cbfb in skeletal development. *Oral Sci Int*. 2015;12(1):1–4.
- Pratap J, Lian JB, Javed A, et al. Regulatory roles of Runx2 in metastatic tumor and cancer cell interactions with bone. *Cancer Metastasis Rev.* 2006;25(4):589–600.
- 34. Guo ZJ, Yang L, Qian F, et al. Transcription factor RUNX2 upregulates chemokine receptor CXCR4 to promote invasive and metastatic potentials of human gastric cancer. *Oncotarget*. 2016;7(15): 20999–21012.
- Sancisi V, Borettini G, Maramotti S, et al. Runx2 isoform I controls a panel of proinvasive genes driving aggressiveness of papillary thyroid carcinomas. J Clin Endocrinol Metab. 2012;97(10):E2006–E2015.
- Colden M, Dar AA, Saini S, et al. MicroRNA-466 inhibits tumor growth and bone metastasis in prostate cancer by direct regulation of osteogenic transcription factor RUNX2. *Cell Death Dis.* 2017;8(1):e2572.
- Zhang Y, Gao Y, Cai L, et al. MicroRNA-221 is involved in the regulation of osteoporosis through regulates RUNX2 protein expression and osteoblast differentiation. *Am J Transl Res.* 2017;9(1):126–135.
- Tu B, Liu S, Yu B, et al. miR-203 inhibits the traumatic heterotopic ossification by targeting Runx2. *Cell Death Dis*. 2016;7(10):e2436.
- Sheppard K, Kinross KM, Solomon B, Pearson RB, Phillips WA. Targeting PI3 kinase/AKT/mTOR signaling in cancer. *Crit Rev Oncog.* 2012;17(1):69–95.

- Lim HJ, Crowe P, Yang JL. Current clinical regulation of PI3K/PTEN/ Akt/mTOR signalling in treatment of human cancer. J Cancer Res Clin Oncol. 2015;141(4):671–689.
- Tandon M, Chen Z, Pratap J. Runx2 activates PI3K/Akt signaling via mTORC2 regulation in invasive breast cancer cells. *Breast Cancer Res.* 2014;16(1):R16.
- Tandon M, Chen Z, Pratap J. Role of Runx2 in crosstalk between Mek/Erk and PI3K/Akt signaling in MCF-10A cells. *J Cell Biochem*. 2014;115(12):2208–2217.

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