

miR-29a suppresses growth and metastasis in papillary thyroid carcinoma by targeting AKT3

Rui Li¹ · Jia Liu² · Qun Li² · Guang Chen² · Xiaofang Yu¹

Received: 15 August 2015 / Accepted: 27 September 2015 / Published online: 19 October 2015
© International Society of Oncology and BioMarkers (ISOBM) 2015

Abstract MicroRNA-29a (miR-29a) has been reported to play important roles in tumor initiation, development, and metastasis in various cancers. However, the biological function and potential mechanisms of miR-29a in papillary thyroid carcinoma (PTC) remain unclear. In the present study, we discovered that miR-29a was frequently downregulated in PTC tissues, and its expression was significantly associated with tumor size, TNM stage, and lymph node metastasis. Functional assays showed that overexpression of miR-29a markedly suppressed PTC cell proliferation, migration, and invasion and promoted PTC apoptosis and cell cycle arrest at G0/G1 phase. In vivo, miR-29a overexpression decreased tumor growth in a xenograft mouse model. Luciferase reporter assay showed that miR-29a can directly bind to the 3' untranslated region (UTR) of AKT3 in PTC cells. Overexpression of miR-29a obviously decreased AKT3 expression, thereby suppressing phosphatidylinositol 3-kinase (PI3K)/AKT pathway activation. We also confirmed that AKT3 expression was increased in PTC tissue and was inversely correlated miR-29a expression in PTC tissues. In addition, downregulation of AKT3 by siRNA mimicked the effects of miR-29a overexpression, and upregulation of AKT3 partially reversed the inhibitory effects of miR-29a. These results suggested that

miR-29a could act as a tumor suppressor in PTC by targeting AKT3 and that miR-29a may potentially serve as an anti-tumor agent in the treatment of PTC.

Keywords Papillary thyroid carcinoma · miR-29a · AKT3 · Proliferation

Introduction

Thyroid cancer (TC) is the most prevalent endocrine neoplasm and has had a steadily increasing incidence over the last several decades [1]. Papillary thyroid carcinoma (PTC), referred to as a differentiated neoplasia, is the most prevalent type of tumor among thyroid malignancies, accounting for approximately 80–90 % of all TC cases [1, 2]. Most patients with PTC have a favorable prognosis, but the overall recurrence rates may be as high as 35 % [3] since clinical and biological behaviors cannot be properly predicted. Therefore, it is an urgent need to understand the molecular mechanisms of PTC progression for finding novel diagnostic, prognostic and therapeutic strategies for this disease.

MicroRNAs (miRNAs) are a class of small non-coding RNAs (19–25 nucleotides) that regulate gene expression at the transcriptional or posttranscriptional level by binding to the complementary 3'-untranslated region (3'-UTR) of messenger RNAs (mRNAs) [4, 5]. Accumulating evidence showed that miRNAs regulate a variety of basic physiological processes, such as cell differentiation, proliferation, and survival, apoptosis, migration, and invasion [6, 7]. MiRNAs have been reported to be associated with cancer development and progression through regulation of cellular proliferation, differentiation, and apoptosis [8–10]. It has been shown that miRNAs can function as either oncogenes or tumor suppressors according to the roles of their target genes [4, 7, 9].

Guang Chen and Xiaofang Yu contributed equally to this paper.

✉ Guang Chen
chenguang518@aliyun.com

✉ Xiaofang Yu
yuxiaofang1542@sina.cn

¹ Institute of Virology and AIDS Research, The First Hospital of Jilin University, Changchun 130021, China

² Department of Thyroid Surgery, The First Hospital of Jilin University, Changchun 130021, China

Therefore, analysis of the miRNAs and the related target genes may provide unique insight into tumorigenesis and new strategies to improve the diagnosis or treatment of PTC.

miR-29a is a member of miR-29s family, which is a conserved family of miRNA [11]. Decreased expression of miR-29s has been described in multiple cancers, such as gastric cancer [12], pancreatic cancer [13] and prostate cancer [14]. In contrast, upregulation of miR-29s was reported in breast cancer [15], nasopharyngeal carcinoma [16], glioma [17] and acute myeloid leukemia [18]. These studies suggested that miR-29a functions as an oncogenic or a tumor-suppressive miRNA in different cancers. However, the clinical significance and its role and underlying molecular mechanism in PTC remain unclear. Therefore, in the present study, we analyzed the association of miR-29a expression with clinicopathologic features in patients suffering PTC and investigate the functional role of miR-29a and potential mechanism of PTC by several in vitro experiments and tumor growth of xenograft in vivo.

Materials and method

Tissue sample

Thirty pairs of human PTC and adjacent normal tissues were harvested at Department of Thyroid Surgery, the First Hospital of Jilin University (Changchun, China) from March 2010 to March 2014. Tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until use. None of the patients had received any preoperative treatment. The characteristics of patients are described in Table 1. Informed consent was obtained from all patients. This study was approved by the Ethic Committee of Jilin University (Changchun, China).

Cell culture

The human PTC cell line, K1 cells were obtained from the Chinese Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Carlsbad, CA) containing with 10 % fetal bovine serum (FBS, Life Technologies, Inc., Grand Island, NY, USA), 100 U/ml penicillin (Sigma Aldrich, St. Louis, MO, USA), or 100 mg/ml streptomycin (Sigma) at 37°C and 5 % CO_2 under a humidified incubator.

Cell transfection

miR-29a mimics (miR-29a) and corresponding miRNA control (miR-Ctrl) were brought from Qiagen (Frederick, MD, USA). siRNA against AKT3 (si-AKT3), siRNA control (si-Ctrl), and overexpression AKT3 plasmid were obtained from GenePharma (Shanghai, China). These molecular productions were transiently transfected into K1 cells

using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Transfection efficiencies were determined in every experiment at 48 h after transfection.

RNA extraction and quantitative reverse-transcription PCR

Total RNA was extracted from cultured cells and tissue using TRIzol reagent (Life Technologies, Inc., USA) according to manufacturer's instructions. PCR primers for miR-29a and U6 snRNA were brought from RiBoBio (Guangzhou, China). For miR-29a expression, total RNA was reverse-transcribed into cDNA using the Universal cDNA synthesis kit from Exiqon (Woburn, MA, USA) according to the manufacturer's instructions. Quantitative PCR was performed using the Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on the Stratagene Mx3005P real-time PCR System (Agilent StrataGene, USA). For AKT3 mRNA expression, total RNA was reverse-transcribed into cDNA using PrimeScriptTM RT reagent Kit (TaKaRa, Dalin, China). Quantitative PCR was carried out using SYBR green PCR master mix (TaKaRa) under the Stratagene Mx3005P real-time PCR System (Agilent StrataGene). Primers of AKT3 and GAPDH were: AKT3 forward: 5'-ACCGCACACGTTTCTATGGT-3', reverse: 5'-CCCTCCACCAAGGCGTTTAT-3'; GAPDH: forward 5'-GGATTTGGTCGTATTGGG-3', reverse: 5'-GTGGCTGGGCTCTACTTC-3'. U6 snRNA or GAPDH expression was used as an endogenous control. Relative gene

Table 1 Correlation between clinicopathological features and miR-29a expression in PTC tissues

Variables	No. of cases	miR-29a expression		P value
		Low (n %)	High (n %)	
Age (years)				$P > 0.05$
<60	18	8(44.4)	10(55.6)	
≥ 60	12	6(50.0)	6(50.0)	
Gender				$P > 0.05$
Male	17	8(46.2)	9(53.8)	
Female	13	6(46.1)	7(53.9)	
TNM stage				$P < 0.01$
I-II	18	5(27.8)	17(72.2)	
III-IV	12	9(75.0)	3(25.0)	
Tumor size				$P < 0.01$
<5	21	7(33.3)	16(66.7)	
≥ 5	9	7(77.8)	3(22.2)	
Lymph node metastasis				$P < 0.01$
No	20	5(25.0)	15(75.0)	
Yes	10	9(90.0)	1(10.0)	

expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. All quantitative reverse-transcription PCR (qRT-PCR) reactions were run in triplicate.

Cell proliferation assay

For cell proliferation assay, 2×10^3 transfected cells/well was seeded in 96-well plates and were continually cultured for 72 h. At different time point (24, 48, and 72 h), 10 μ l of 0.5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) was added to each well. Then, cells were incubated for 4 h at 37 °C, the medium removed, and precipitated formazan dissolved in 150 μ l DMSO (Sigma). After shaking for 20 min, absorbance was detected at 490 nm using a biorad iMark Microplate Absorbance Reader (BD Biosciences, Mansfield, MA, USA).

Cell cycle and apoptosis assays

Cell cycle and apoptosis were examined by flow cytometry at 48 h posttransfection. For cell cycle assay, transfected cells were fixed in 10 μ l ice-cold ethanol for at least 2 h and then washed twice with PBS and resuspended in PBS containing 0.2 % Triton X-100 for permeabilization and propidium iodide (PI, Sigma) staining. After incubation for 30 min, the cells were analyzed using FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed using CellQuest software (BD Biosciences). Each experiment was performed in triplicate.

For cell apoptosis, transfected cells were double-stained with fluorescein (FITC)-conjugated Annexin V and propidium iodide (FITC-Annexin V/PI) (BD Biosciences) and analyzed on a FACSCalibur flow cytometer (BD Biosciences) to determine rate of apoptosis.

Cell migration and invasion assays

Cell migration assay was performed using wound healing assay. Briefly, transfected cells were seeded in 6-well culture plates. When cells reached 90–95 % confluence, they were scratched with a micropipette tip in the cell monolayer. The cells were then allowed to migrate in an incubator. At 0 and 24 h, three fields of the wound area were photographed with a light microscope ($\times 100$, Olympus, Tokyo, Japan). For each image, the area that was uncovered by cells was analyzed by using Image Pro Plus 4.5.1 software. For the invasion assays, transfected cells in serum-free DMEM medium were placed into the top chamber coated with Matrigel (BD Biosciences, San Jose, CA, USA) and incubated at 37 °C for 4 h, allowing it to solidify. DMEM medium containing 10 % FBS were added to the lower chambers as a chemoattractant. After incubation at 37 °C for 48 h, cells adhering to the lower membrane were stained with 0.1 % crystal violet in 20 % methanol, imaged,

and then five randomly fields of each filter were manually counted under light microscope ($\times 200$, Olympus, Tokyo, Japan).

Vector construction and luciferase reporter assay

The 3' UTR of AKT3 containing the miR-29a recognition sequence was amplified by PCR using genomic DNA. The PCR product was then cloned into the pGL3-control vector (Ambion, Austin, TX, USA) at the NheI and XhoI restriction sites. A mutant AKT3 3' UTR was synthesized using a site-directed mutagenesis kit (TaKaRa, Toyko, Japan).

For luciferase activity assay, K1 cells were co-transfected with wild-type (WT) or mutant (Mut) 3'-UTR of AKT3 and miR-29a or miR-Ctrl and were cultured for 48 h. Then, the dual luciferase activities were examined using the Dual-Luciferase Reporter Assay System (Promega, WI, USA), according to the manufacturer's instructions. Normalized data were calculated as the ratio of Renilla/firefly luciferase activities.

Western blotting analysis

Protein extracts were obtained from cultured cells or tissues through a RIPA buffer (Beyotime, Beijing, China). The total concentration of protein was measured using a bicinchoninic acid (BCA) protein assay kit (Boster, Beijing, China). Equal amounts of proteins (30 μ g each lane) were separated on 8–12 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes (Bio-Rad, Munich, Germany). Then, membranes were blocked with 5 % non-fat milk in Tris-buffered saline for 1 h at room temperature, followed by probing with antibodies for AKT3 (1:1000 dilution; Cell signaling, Beverly, MA, USA), phospho-Akt (p-Akt) (Ser473) (1:1000 dilution; Cell Signaling), and GAPDH (1:5000 dilution; Cell Signaling), which was used as a loading control. The membranes were incubated with the appropriate HRP-conjugated IgG (anti-rabbit 1:3000 dilution; Cell Signaling, or anti-mouse 1:10,000 dilution; Santa Cruz Biotechnology), and proteins were detected by with chemiluminescent detection system (ECL, Thermo Scientific, Rockford, IL, USA).

Tumorigenicity assay in nude mice

Twenty BALB/c male nude mice (4–6 weeks, 18–22 g) were brought from Changchun Biological Institute (Changchun, China) and maintained under specific pathogen-free (SPF) conditions. All animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Jilin University (Changchun, China).

K1 cells (2×10^6) stably expressing miR-29a or miR-Ctrl were injected subcutaneously in the flanks of nude mice ($n =$

10). Tumor growth was determined every 5 days for 30 days by measuring the length (L), width (W), and height (H) with calipers and using the formula $\text{Volume (V)} = [\pi/6 \times L \times W \times H]$. Thirty days after inoculation, the mice were killed and tumor tissues were striped and weighted. Parts of the tumor tissues were snap frozen in liquid nitrogen and stored at -80°C for analysis for miR-29a and AKT3 expression.

Statistical analysis

The results were expressed as the mean \pm standard deviation (SD) of at least three separate experiments. Statistical significance was analyzed using Student's *t* test or one-way ANOVA. All statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) and the GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA). Differences with $P < 0.05$ were considered statistically significant.

Results

MiR-29a is downregulated in human PTC tissues

We assessed miR-29a expression by qRT-PCR in the 30 PTC tissues and matched adjacent normal tissues. It was found that the relative expression of miR-29a in PTC tissues was significantly lower than that those of their matched adjacent normal tissues ($P < 0.01$) (Fig. 1). To further investigate the clinicopathological significance of miR-29a level in PTC patients, the median value (0.59) of all 30 PTC samples were chosen as the cutoff point for separating tumors with low relative level of miR-29a (< 0.59 , 14 cases) and high relative level of miR-29a group (> 0.59 , 16 cases). Then, correlations between miR-29a expression and clinicopathologic parameters were analyzed by the chi-square test. Correlation analysis showed that miR-

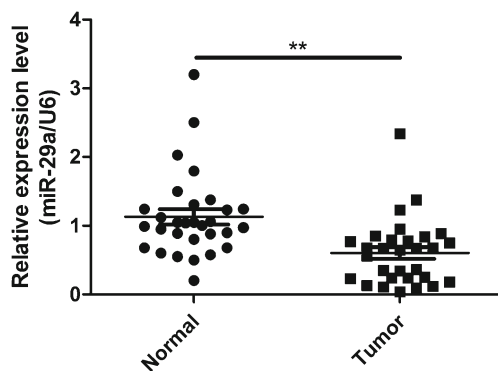


Fig. 1 miR-29a is downregulated in human PTC tissues. Relative levels of miR-29a in PTC tissue ($n=30$) and matched adjacent normal tissues were determined by quantitative RT-PCR (qRT-PCR). U6 was used as loading control. $**P < 0.01$ versus normal tissues

29a expression was not significantly associated with age and gender, while the level of miR-29a negatively correlated with TNM stage ($P < 0.01$), tumor size ($P < 0.01$), and lymph node metastasis ($P < 0.01$), which are all indicators of poor prognosis (Table 1). These results suggested that miR-29a might involve in PTC procession.

miR-29a inhibits PTC cell proliferation and induces and apoptosis

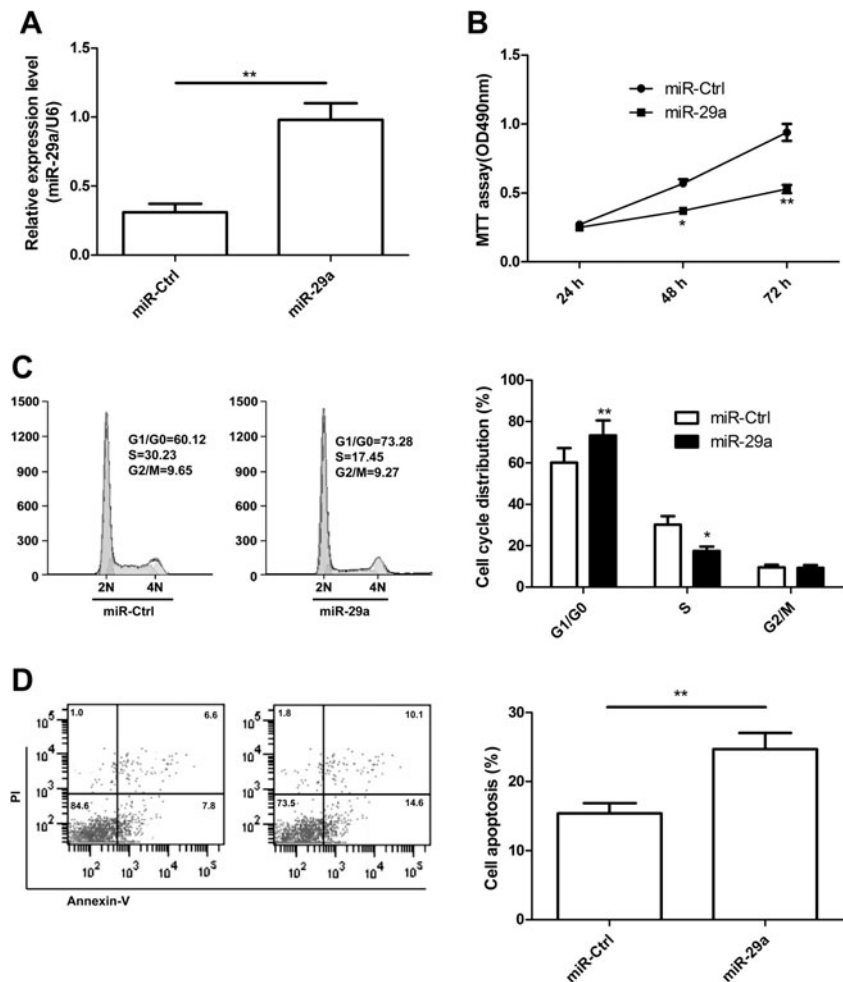
To study the biological role of miR-29a in PTC, miR-29a stably expressing cell lines of K1 was established by transfection of miR-29a mimic. Successful increase of miR-29a expression in K1 cells was confirmed by qRT-PCR (Fig. 2a). Then, cell proliferation, cycle, and apoptosis were determined in K1 cells transfected with miR-29a mimic or miR-Ctrl. MTT assay showed that restoration of miR-29a significantly inhibited cell proliferation in K1 cells (Fig. 2b) compared to miR-Ctrl group. Cell cycle assay showed that restoration of miR-29a in K1 cells significantly decreased the percentage of S phase and increased percentage of G0/G1 phase compared to miR-Ctrl group ($P < 0.05$, Fig. 2c). Cell apoptosis assay revealed that restoration of miR-29a in K1 cells significantly induced cells apoptosis compared to miR-Ctrl group ($P < 0.05$, Fig. 2d). Thus, these results indicated that miR-29a can efficiently inhibited PTC cell growth by inhibiting cell proliferation and inducing cell apoptosis.

miR-29a inhibits PTC cells invasion and migration

We next investigated the role of miR-29a in regulating PTC cell migration and invasion by wounding heal and invasion chamber assay. It was found that restoration of miR-29a significantly inhibited cell migration (Fig. 3a) and invasion (Fig. 3b) capacities of K1 cells.

AKT3 is a direct target of miR-29a Potential targets of miR-29a were predicted using bioinformatic databases such as TargetScan, miRanda and PicTar. AKT3 has been chosen as study object since it plays a central role in the PI3K/AKT signaling pathway and involves in multiple cellular functions [19]. To verify whether AKT3 is a direct target of miR-29a in PTC, a human AKT3 3' UTR fragment containing the binding sites of miR-29a (Fig. 4a) or the mutant sites was cloned into the pGL3 vector, then along with miR-29a mimic or miR-Ctrl were co-transfected into K1 cells and cultured for 48 h, and luciferase activities were determined. It was found that overexpression of miR-29a expression obviously suppressed the luciferase activity of wide-type AKT3 site, but the activity of the mutant AKT3 site was not change (Fig. 4b), suggesting that AKT3 is a directly target of miR-29a. Then, qRT-PCR and western blotting analysis confirmed that overexpression

Fig. 2 miR-29a inhibits proliferation and induces apoptosis in PTC cells. **a** qRT-PCR determined miR-29a expression in K1 cells transfected with miR-29a mimic or miR-Ctrl. **b–d** Cell proliferation (**b**), cycle procession (**c**), and apoptosis (**d**) were determined in K1 cells transfected with miR-29a mimic or miR-Ctrl. * $P < 0.05$, ** $P < 0.01$ versus miR-Ctrl



of miR-29a drastically inhibited AKT3 expression on mRNA level (Fig. 4c) and protein level (Fig. 4d) in K1 cells. In addition, we also found that overexpression of miR-29a also inhibited phospho-Akt (p-Akt) expression, suggesting that miR-29a could inhibit PI3K/AKT signal pathway activation (Fig. 4d). These results indicated that miR-29a can bind directly to AKT3 and inhibits its expression.

miR-29a expression is inversely correlated with AKT3 expression in PTC tissues. Knowing AKT3 was the target of miR-29a, we investigated the expression of AKT3 in PTC specimens and their matched adjacent normal tissues from 30 PTC patients by qRT-PCR. It was found that AKT3 mRNA expression levels were increased in PTC tissues compared to matched adjacent normal tissues (Fig. 5a) and was negatively correlated with miR-29a expression in PTC tissues (Fig. 5b; $r = -0.518$, $P < 0.01$).

Downregulation of Akt3 had similar effects of miR-29a

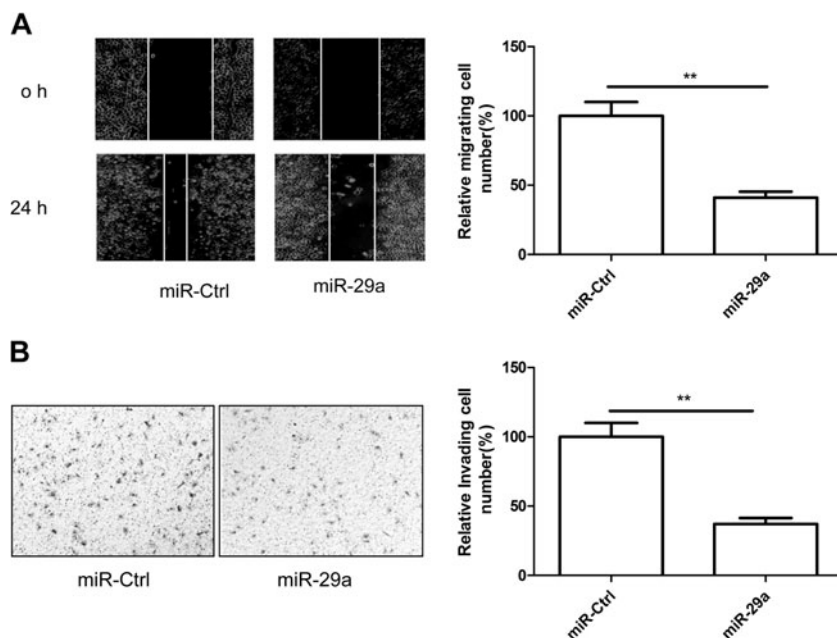
To investigate the biological role of AKT3 in PTC, K1 cells were transfected with si-AKT3 or the si-Ctrl. The knockdown

efficiency of AKT3 was verified by qRT-PCR and western blot (Fig. 6a, b). After si-AKT3 treatment, MTT, apoptosis, wound healing, and invasion assays were performed. As presented in Fig. 6c–f, downregulation of AKT3 by si-AKT3 in K1 cells inhibited cell proliferation and induced apoptosis, as well as suppressed cell migration and invasion capabilities. In other words, reduction of AKT3 mimicked the effect of miR-29a overexpression.

Overexpression of AKT3 rescues the effects of miR-29a

To investigate the functional relevance of AKT3 targeting by miR-29a, we assessed whether overexpression of AKT3 could rescue the effects of miR-29a on PTC cells proliferation, apoptosis, migration, and invasion. To this end, overexpression of AKT3 plasmid was transfected into K1 cells along with miR-29a mimics or miR-Ctrl. It was found that overexpression of AKT3 plasmid combination with miR-29a could increase AKT3 expression on mRNA level (Fig. 7a) and protein level (Fig. 7b) compared to miR-29a alone. In addition, our results also showed that overexpression of AKT3 in K1 cells

Fig. 3 miR-29a inhibits migration and invasion of PTC cells. **a** Cell migration was determined in K1 cells transfected with miR-29a mimic or miR-Ctrl by wound healing assay. **b** Cell invasion was determined in K1 cells transfected with miR-29a mimic or miR-Ctrl by invasion chamber assay. $P < 0.05$, $**P < 0.01$ versus miR-Ctrl



could reverse the effect of miR-29a on cell proliferation, apoptosis, migration, and invasion (Fig. 7c–f). These data indicated that miR-29a exerts inhibition effect on PTC growth and metastasis partially by targeting AKT3.

miR-29a suppresses PTC growth in vivo Having been shown in vitro that miR-29a possessed tumor-suppressive activities, we undertook to evaluate its role in vivo. To this aim,

the human K1 cells stably expression miR-29a or miR-Ctrl was implanted subcutaneously into nude mice to allow tumor formation. Tumors grew slower in the K1/miR-29a group than that of the K1/miR-Ctrl group (Fig. 8a). At the 30th day post-injection, the mice were killed, and tumor tissue was stripped. A significant decrease in tumor size (Fig. 8b) and weight (Fig. 8c) was observed in mice injected with K1/miR-29a compared to the group injected with K1/ miR-Ctrl.

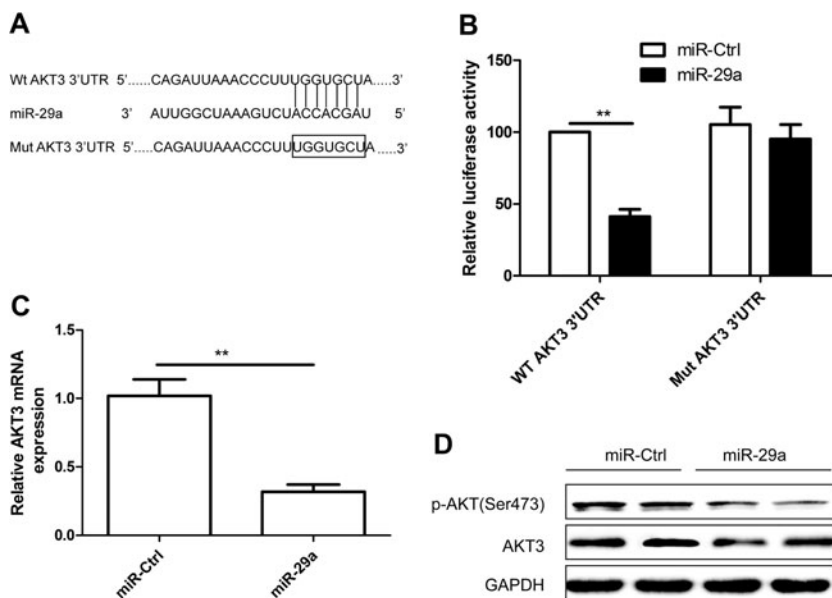


Fig. 4 AKT3 is a direct target of miR-29a. **a** The predicted binding sites for miR-29a in the 3'UTR of AKT3 and the mutations in the binding sites are shown. **b** Relative luciferase activity was determined in K1 cells co-transfection with wide-type or mutant-type 3' UTR AKT3 reporter plasmids and miR-29a or miR-Ctrl. *Wt* wide-type, *Mut* mutant-type. **c** AKT3

mRNA expression in K1 cells transfected with miR-29a or miR-Ctrl were determined by qRT-PCR. **d** AKT3 and p-AKT protein expression in K1 cells transfected with miR-29a mimic or miR-Ctrl were determined by Western blot. qRT-PCR and Western blot data were normalized to GAPDH. $*P < 0.05$, $**P < 0.01$ versus miR-Ctrl

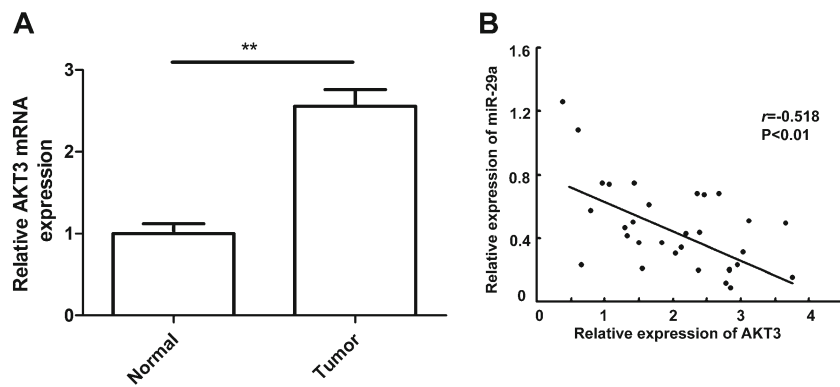


Fig. 5 miR-29a expression is inversely correlated with AKT3 expression in PTC tissues. **a** AKT3 mRNA expression in 30 cases of PTC tissue and matched normal tissues were determined by qRT-PCR. GAPDH was used

as an internal control. * $P < 0.05$, ** $P < 0.01$ versus normal tissues. **b** The reverse relationship between AKT3 mRNA expression and miR-29a expression was explored by Spearman's correlation

Furthermore, we also determined miR-29a and AKT3 expression in tumor tissue. We found that the miR-29a expression significantly increased (Fig. 8d), whereas AKT3 expression remarkably decreased both on mRNA level (Fig. 8e) and protein level (Fig. 8f) in K1/miR-29a group compared to K1/miR-Ctrl group. These results suggest that miR-29a suppress PTC growth in vivo by targeting AKT3.

Discussion

It is well known that miRNAs play critical roles as either oncogenes or tumor suppressors in human various cancers including thyroid cancer [20]. Recently, a number of miRNAs involved in PTC cell proliferation, migration, and invasion have been identified [21–24]. For instance, Ma et al. found that miR-34a functions, as an oncogene in PTC,

can promote cell proliferation and inhibit apoptosis in papillary thyroid carcinoma by targeting growth arrest specific1 (GAS1) via PI3K/Akt/Bad pathway [25]. Liu et al. reported that miR-204-5p can inhibit cell proliferation and colony formation, block cell cycle progression, and enhance apoptosis in vitro and suppress tumorigenicity in vivo by targeting IGFBP5 [26]. Gu et al. found that miR-539 inhibited cell migration and invasion in human thyroid cancer cells by targeting CARMA1 (CARD domain and MAGUK domain-containing protein-1) [27]. Guan et al. showed that miR-144 inhibited thyroid cancer cell invasion and migration via directly targeting ZEB1 and ZEB2 [28]. Here, we found that miR-29a expression is downregulated in PTC tissue, and that restoration of miR-29a inhibited proliferation, migration, and invasion, induced cell apoptosis in vitro, and suppressed tumor growth in nude mice model. These findings suggested that miR-29a might act as a therapy agent for treatment of PTC.

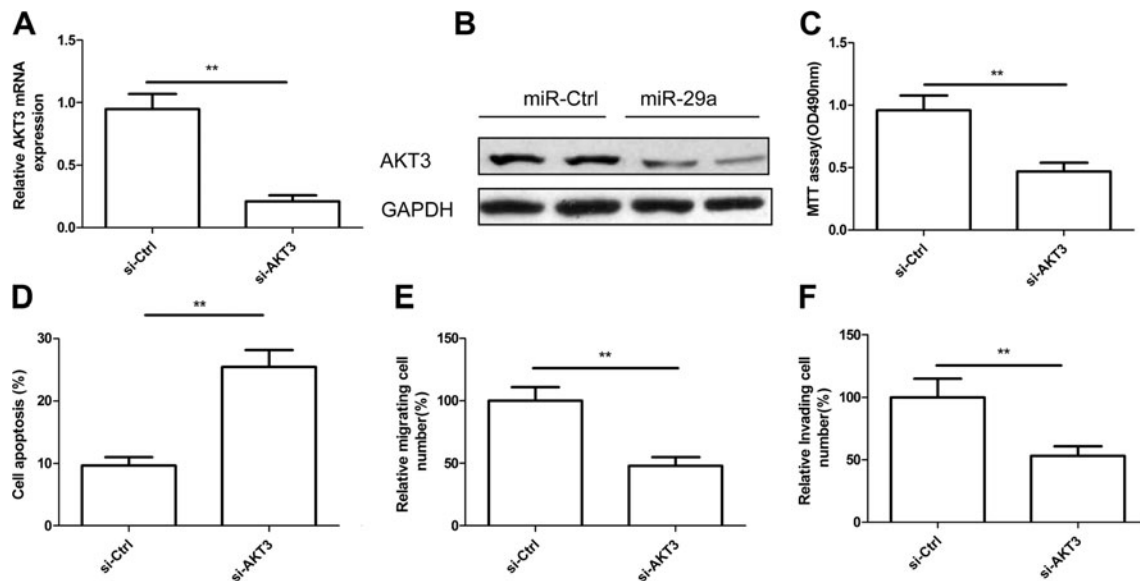


Fig. 6 Downregulation of AKT3 mimicked effects of overexpression miR-29a. **a**, **b** AKT3 expression on mRNA level (**a**) and protein level (**b**) was detected in K1 cells transfected with si-AKT3 or si-Ctrl. GAPDH

was used as an internal control. **c–f** Cell proliferation (**c**), apoptosis (**d**), migration (**e**), and invasion (**f**) were determined in K1 cells transfected with si-AKT3 or si-Ctrl. * $P < 0.05$, ** $P < 0.01$ versus si-NC group

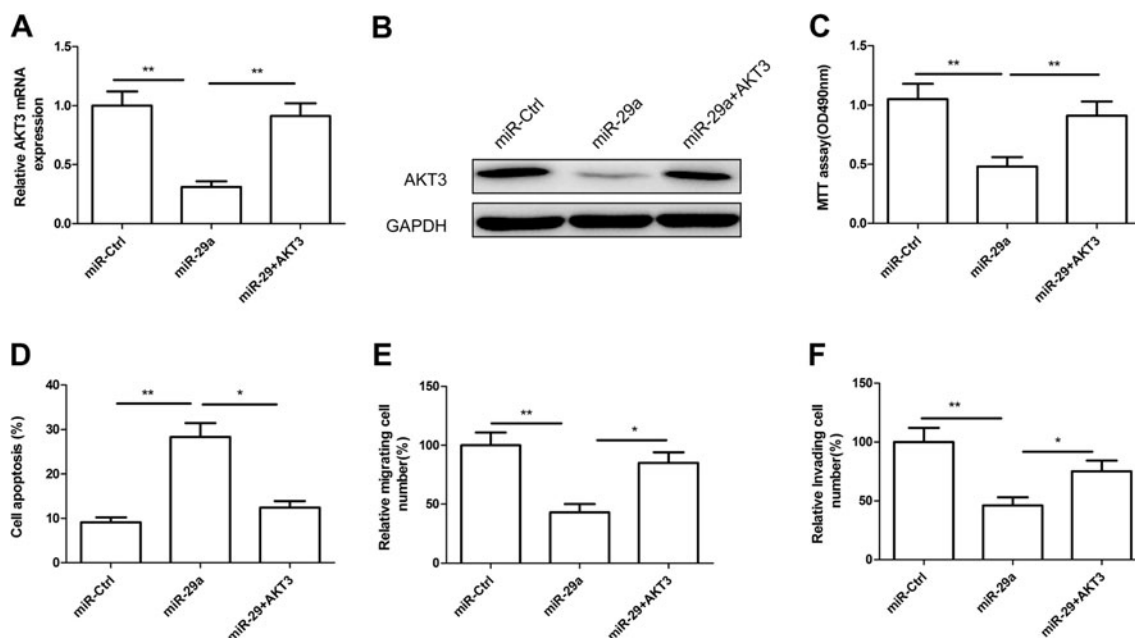


Fig. 7 Overexpression of AKT3 rescues the effects of miR-29a. **a, b** AKT3 expression on mRNA level (**a**) and protein level (**b**) in K1 cells co-transfected with AKT3 overexpression plasmid and miR-29a mimic or miR-Ctrl. GAPDH was used as an internal control. **c-f** Cell proliferation

(**c**), apoptosis (**d**), migration (**e**), and invasion (**f**) were determined in K1 cells transfected with miR-29a mimic with/without AKT3 overexpression plasmid. * $P < 0.05$, ** $P < 0.01$ versus miR-29a

miR-29a is one of the members of miR-29 family, which also includes miR-29b and miR-29c [29]. Accumulating evidences have shown that miR-29 family was aberrantly expressed in several types of human cancers and was involved in complex regulatory process by targeting multiple factors associated with several signal

pathways including PI3K/AKT pathway and Wnt/ β -catenin signaling pathway, suggesting its key role in carcinogenesis and cancer progression [11, 30–33]. Recent studies have found that miR-29a is upregulated in breast cancer [15], nasopharyngeal carcinoma [16], glioma [17], and acute myeloid leukemia [18]. However, some

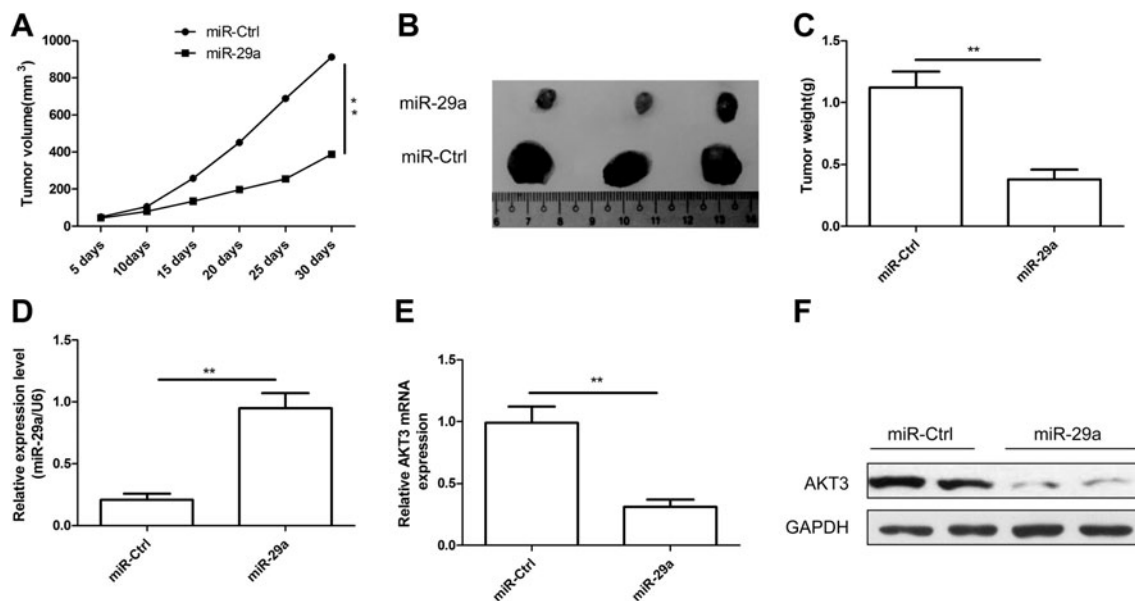


Fig. 8 miR-29a inhibits PTC growth in vivo. **a** Growth curves for tumor volumes were established based on the tumor volume measured every 5 days until 30 days. **b** Graphs of the tumor tissue. **c** Tumor tissues weights. **d** miR-29a expression in tumor tissue were determined by

qRT-PCR. U6 was used to an internal control. **e, f** AKT3 expression on mRNA level (**e**) and protein level (**f**) in tumor tissue were determined. GAPDH was used to an internal control. * $P < 0.05$, ** $P < 0.01$ versus miR-Ctrl

researches reveal that miR-29a is downregulated in gastric cancer [12], pancreatic cancer [13], and prostate cancer [14]. These inconsistent findings indicate that dysregulation of miR-29a in various cancers may be dependent on the cellular microenvironment, details tumor type, and its target genes, which need further investigation in different cancers. Here, to investigate the potential role of miR-29a in PTC, we investigated the expression of miR-29a in 30 PTC samples and their paired adjacent normal tissues by qRT-PCR. We found that miR-29a was significantly downregulated in PTC clinical specimens, and its expression was negatively correlated with TNM stage, tumor size, and lymph node metastasis. Function assay showed that miR-29a inhibited PTC cell growth, migration, and invasion in vitro and suppressed tumor growth in vivo partially by targeting AKT3. Based on these findings, we suggested that miR-29a might act as a tumor suppressor in PTC.

miRNAs usually exert their biologic functions by regulating targeting gene expression [34]. To further investigate possible functional mechanisms of miR-29a in PTC and to identify target genes of miR-29a, in this study, we used three bioinformatics algorithms to predict gene targets for miR-29a and found that AKT3 contains a highly conserved miR-29a binding site on the 3'UTR. AKT3 is one of the members of AKT family, which also includes AKT1 and AKT2 [6]. AKT is a central protein mediating signals from receptor tyrosine kinases and phosphatidylinositol 3-kinase (PI3K) [35]. As a key component of the PI3K/AKT pathway, AKT regulates multiple biological processes, such as cell proliferation, apoptosis, cell cycle progression, migration, and invasion [36, 37]. It has been shown that AKT pathway plays important roles in the initiation and development of various cancers, including thyroid cancer [38]. In our current study, we demonstrated an important molecular association between miR-29a and AKT3. The results of luciferase activity assay showed that miR-29a could directly target the 3'-UTRs of AKT3. Western blot assay showed that miR-29a could inhibit AKT3 and p-AKT protein expression. Downregulation of AKT3 had similar effects of miR-29a overexpression. Finally, overexpression of AKT3 could partially rescue the miR-29a-induced effects. These findings suggested that miR-29a exerted suppressive effect on PTC growth and metastasis partially by targeting AKT3.

In conclusion, the present study first showed that miR-29a was significantly decreased in PTC tissues, and its expression was negatively correlated with TNM stage, tumor size, and lymph node metastasis, and that miR-29a inhibited PTC cell proliferation, migration, and invasion, induced cell apoptosis, as well as suppressed PTC tumor growth in vivo via targeting AKT3. These findings suggested that miR-29a might act as a tumor suppressor in PTC and that restoration of miR-29a might have therapeutic potential for PTC.

Compliance with ethical standards

Conflicts of interest None

References

1. Brown RL, de Souza JA, Cohen EE. Thyroid cancer: burden of illness and management of disease. *J Cancer*. 2011;2:193–9.
2. Pitoia F, Bueno F, Urciuoli C, Abelleira E, Cross G, Tuttle RM. Outcomes of patients with differentiated thyroid cancer risk-stratified according to the American thyroid association and Latin American thyroid society risk of recurrence classification systems. *Thyroid Off J Am Thyroid Assoc*. 2013;23:1401–7.
3. Grant CS. Recurrence of papillary thyroid cancer after optimized surgery. *Gland Surg*. 2015;4:52–62.
4. Brennecke J, Cohen SM. Towards a complete description of the microma complement of animal genomes. *Genome Biol*. 2003;4:228.
5. Ambros V. The functions of animal micromas. *Nature*. 2004;431:350–5.
6. Bartel DP. Micromas: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116:281–97.
7. Carthew RW, Sontheimer EJ. Origins and mechanisms of mirnas and simas. *Cell*. 2009;136:642–55.
8. Esquela-Kerscher A, Slack FJ. Oncomirs - micromas with a role in cancer. *Nat Rev Cancer*. 2006;6:259–69.
9. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D. Micromas expression profiles classify human cancers. *Nature*. 2005;435:834–8.
10. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A micromas expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A*. 2006;103:2257–61.
11. Wang Y, Zhang X, Li H, Yu J, Ren X. The role of mirna-29 family in cancer. *Eur J Cell Biol*. 2013;92:123–8.
12. Cui Y, Su WY, Xing J, Wang YC, Wang P, Chen XY, et al. Mir-29a inhibits cell proliferation and induces cell cycle arrest through the downregulation of p42.3 in human gastric cancer. *PLoS One*. 2011;6:e25872.
13. Trehoux S, Lahdaoui F, Delpu Y, Renaud F, Leteurtre E, Torrisani J, et al. Micro-rnas mir-29a and mir-330-5p function as tumor suppressors by targeting the muc1 mucin in pancreatic cancer cells. *Biochim Biophys Acta*. 1853;2015:2392–403.
14. Li Y, Kong D, Ahmad A, Bao B, Dyson G, Sarkar FH. Epigenetic deregulation of mir-29a and mir-1256 by isoflavone contributes to the inhibition of prostate cancer cell growth and invasion. *Epigenetics*. 2012;7:940–9.
15. Zhong S, Li W, Chen Z, Xu J, Zhao J. Mir-222 and mir-29a contribute to the drug-resistance of breast cancer cells. *Gene*. 2013;531:8–14.
16. Qiu F, Sun R, Deng N, Guo T, Cao Y, Yu Y, et al. Mir-29a/b enhances cell migration and invasion in nasopharyngeal carcinoma progression by regulating sparc and col3a1 gene expression. *PLoS One*. 2015;10:e0120969.
17. Zhao D, Jiang X, Yao C, Zhang L, Liu H, Xia H, et al. Heat shock protein 47 regulated by mir-29a to enhance glioma tumor growth and invasion. *J Neuro-Oncol*. 2014;118:39–47.
18. Zhu C, Wang Y, Kuai W, Sun X, Chen H, Hong Z. Prognostic value of mir-29a expression in pediatric acute myeloid leukemia. *Clin Biochem*. 2013;46:49–53.
19. Kang SS, Kwon T, Kwon DY, Do SI. Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J Biol Chem*. 1999;274:13085–90.

20. de la Chapelle A, Jazdzewski K. Micromas in thyroid cancer. *J Clin Endocrinol Metab.* 2011;96:3326–36.
21. Chiang CH, Hou MF, Hung WC. Up-regulation of mir-182 by beta-catenin in breast cancer increases tumorigenicity and invasiveness by targeting the matrix metalloproteinase inhibitor reck. *Biochim Biophys Acta.* 1830;2013:3067–76.
22. Visone R, Russo L, Pallante P, De Martino I, Ferraro A, Leone V, et al. Micromas (mir)-221 and mir-222, both overexpressed in human thyroid papillary carcinomas, regulate p27kip1 protein levels and cell cycle. *Endocr-Relat Cancer.* 2007;14:791–8.
23. Mardente S, Mari E, Consorti F, Di Gioia C, Negri R, Etna M, et al. Hmgb1 induces the overexpression of mir-222 and mir-221 and increases growth and motility in papillary thyroid cancer cells. *Oncol Rep.* 2012;28:2285–9.
24. Zhang X, Li M, Zuo K, Li D, Ye M, Ding L, et al. Upregulated mir-155 in papillary thyroid carcinoma promotes tumor growth by targeting apc and activating wnt/beta-catenin signaling. *J Clin Endocrinol Metab.* 2013;98:E1305–13.
25. Ma Y, Qin H, Cui Y. Mir-34a targets gas1 to promote cell proliferation and inhibit apoptosis in papillary thyroid carcinoma via pi3k/akt/bad pathway. *Biochem Biophys Res Commun.* 2013;441:958–63.
26. Liu L, Wang J, Li X, Ma J, Shi C, Zhu H, et al. Mir-204-5p suppresses cell proliferation by inhibiting igfbp5 in papillary thyroid carcinoma. *Biochem Biophys Res Commun.* 2015;457:621–6.
27. Gu L, Sun W. Mir-539 inhibits thyroid cancer cell migration and invasion by directly targeting CARMA1. *Biochem Biophys Res Commun.* 2015;464: 1128–33.
28. Guan H, Liang W, Xie Z, Li H, Liu J, Liu L, et al. Down-regulation of mir-144 promotes thyroid cancer cell invasion by targeting zeb1 and zeb2. *Endocrine.* 2015;48:566–74.
29. Jiang H, Zhang G, Wu JH, Jiang CP. Diverse roles of mir-29 in cancer (review). *Oncol Rep.* 2014;31:1509–16.
30. Amodio N, Rossi M, Raimondi L, Pitari MR, Botta C, Tagliaferri P, et al. Mir-29s: a family of epi-mirnas with therapeutic implications in hematologic malignancies. *Oncotarget.* 2015;6:12837–61.
31. Rostas 3rd JW, Pruitt HC, Metge BJ, Mitra A, Bailey SK, Bae S, et al. Microna-29 negatively regulates emt regulator n-myc interactor in breast cancer. *Mol Cancer.* 2014;13:200.
32. Nishikawa R, Goto Y, Kojima S, Enokida H, Chiyomaru T, Kinoshita T, et al. Tumor-suppressive micromna-29s inhibit cancer cell migration and invasion via targeting lamc1 in prostate cancer. *Int J Oncol.* 2014;45:401–10.
33. Tan M, Wu J, Cai Y. Suppression of wnt signaling by the mir-29 family is mediated by demethylation of wif-1 in non-small-cell lung cancer. *Biochem Biophys Res Commun.* 2013;438:673–9.
34. Hers I, Vincent EE, Tavares JM. Akt signalling in health and disease. *Cell Signal.* 2011;23:1515–27.
35. Guo H, German P, Bai S, Barnes S, Guo W, Qi X, et al. The pi3k/akt pathway and renal cell carcinoma. *J Genet Genomics = Yi Chuan Xue Bao.* 2015;42:343–53.
36. Xia P, Xu XY. Pi3k/akt/mtor signaling pathway in cancer stem cells: from basic research to clinical application. *Am J Cancer Res.* 2015;5:1602–9.
37. Petrulea MS, Plantinga TS, Smit JW, Georgescu CE, Netea-Maier RT. Pi3k/akt/mtor: a promising therapeutic target for non-medullary thyroid carcinoma. *Cancer Treat Rev.* 2015;41:707–13.
38. Xing M. Genetic alterations in the phosphatidylinositol-3 kinase/akt pathway in thyroid cancer. *Thyroid Off J Am Thyroid Assoc.* 2010;20:697–706.