# MYC promotes the development of papillary thyroid carcinoma by inhibiting the expression of lncRNA PAX8-AS1:28

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**Abstract.** As a common malignancy of the endocrine system, papillary thyroid carcinoma (PTC) seriously affects the quality of life of patients. lncRNA PAX8-AS1:28, or lnc-PSD4-1:14 has been reported to be abnormally expressed in PTC. However, the function of PAX8-AS1:28 in PTC is still unknown. Therefore, the present study aimed to investigate the functions of PAX8-AS1:28 in PTC, and to explore the possible mechanisms of action. A total of 38 patients with PTC were included and the normal thyroid follicular epithelial cell line Nthy-ori 3-1 and PTC cell line IHH-4 were also used. MYC and PAX8-AS1:28 overexpression and siRNA silencing in the cell lines were carried out. Expression of PAX8-AS1:28, PAX8 and MYC in tumor tissue, adjacent healthy tissue and different cell lines were detected by qRT-PCR and western blot analysis. Cell proliferation was measured by CCK-8 assay. Expression levels of PAX8-AS1:28 and PAX8 were lower in PTC tumor tissue and PTC cells than those in healthy tissue and normal cells. In contrast, the expression level of MYC was higher in PTC cells than that in normal cells. PAX8-AS1:28 silencing reduced the expression level of PAX8 and promoted tumor cell growth, while PAX8-AS1:28 overexpression increased the expression level of PAX8 and inhibited tumor cell growth. MYC silencing increased expression levels of PAX8-AS1:28 and PAX8 and inhibited tumor cell growth, while MYC overexpression decreased expression levels of PAX8-AS1:28 and PAX8 and promoted tumor cell growth. MYC can promote PTC by inhibiting the expression of lncRNA PAX8-AS1:28.

## Introduction

Apart from medullary carcinoma, all other types of thyroid cancers originate from follicular cells, which form the simple unicellular epithelium of the thyroid (1). In spite of the development of preventive measures, the incidence of thyroid cancer has increased more than 3-fold during the past 3 decades, and this disease tends to affect younger patients (2). As the most common type of thyroid cancer, papillary thyroid carcinoma (PTC) accounts for more than 80% of the total cases of this disease (3). With the development of treatment strategies such as radiotherapy, chemotherapy, thyroid hormone treatment, surgical resection and combined treatment, the 5-year survival rate of patients with PTC has now reached 95% (4). However, due to the unclear pathogenesis, the recurrence rate of this disease is still high (5). More importantly, PTC may occasionally dedifferentiate into more aggressive and lethal thyroid cancers (1). Therefore, the in-depth understanding of the pathogenesis of this disease is highly needed.

Long non-coding RNA, which is also known as lncRNA, is a class of non-coding RNAs with a length longer than 200 nucleotides, which is significantly longer than that of short interfering RNAs, microRNAs and other short RNAs (6). Numerous studies have shown that different lncRNAs have their specific functions in normal biological or pathological processes (7). In addition to this, quite a few of lncRNAs were proven to play essential roles in the development and progression of a variety of human diseases including liver diseases, heart diseases, and different types of human cancer (8-10). A recent study showed that lncRNA PAX8-AS1:28, or lnc-PSD4-1:14, is abnormally expressed in PTC (11), indicating the involvement of lncRNA PAX8-AS1:28 in this disease. However, the role of lnc-PSD4-1:14 in PTC is still unknown.

In the present study, expression of lncRNA PAX8-AS1:28 in PTC tissues and adjacent healthy tissues were detected. The relationships between PAX8-AS1:28, PAX8 and MYC were investigated.

## Materials and methods

*Patients*. A total of 38 patients with PTC were enrolled in our hospital from October 2016 to October 2017. These patients included 13 males and 25 females, and the ages ranged from 20 to 78 years with an average age of 40.2 years. All patients were diagnosed by the standards that have been established by the World Health Organization. Patients with thyroid microcarcinoma were excluded. All patients received surgical resections, and tumor tissues as well as adjacent healthy tissues

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were collected during the surgical operation. All patients had normal thyroid function before surgery, and none of them received radiotherapy or chemotherapy before admission. The Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University approved this study. All patients provided informed consent.

*Cell lines and cell culture*. Normal thyroid follicular epithelial cell line Nthy-ori 3-1 and PTC cell line IHH-4 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured with DMEM containing 10% FBS, 100 mg/ml penicillin G and 100 U/ml streptomycin (all from Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an incubator at 37˚C in 5% $CO_2$.

*Real-time quantitative PCR*. Trizol reagent (Invitrogen; Thermo Fisher Scientific) was used for total RNA extraction. The quality of RNA samples was tested using NanoDrop™ 2000 spectrophotometers (Thermo Fisher Scientific), and only those with an A260/A280 ratio between 1.8 and 2.0 were used in reverse transcription to synthesize cDNA by using Oligo$(dT)_{15}$ (Sangon, Shanghai, China) and AMV reverse transcriptase (Gibco; Thermo Fisher Scientific). TaqMan PCR kit (Thermo Fisher Scientific) was used for PCR reaction. The PCR reaction was performed on Bio-Rad iCycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The following primers were used in the PCR reactions: 5'-GGCTTTGTGCTACTGCTTCA-3' (forward) and 5'-TCTAACCCTCCTGGCTTCCT-3' (reverse) for PAX8-AS1:28; 5'-GATCAGGATAGCTGCCGACT-3' (forward) and 5'-GTTGTACCTGCTCGCCTTTG-3' (reverse) for PAX8; 5'-CAGGAGGCATTGCTGATGAT-3' (forward) and 5'-GAAGGCTGGGGCTCATTT-3' (reverse) for GAPDH; 5'-GCCACGTCTCCACACATCAG-3' (forward) and 5'-TCT TGGCAGCAGGATAGTCCTT-3' (reverse) for MYC. PCR reaction conditions were: 95˚C for 1 min, followed by 40 cycles of 95˚C for 15 sec and 56˚C for 35 sec. Ct values were processed using the $2^{-\Delta\Delta CT}$ method, and relative expression level of each gene was normalized to endogenous control GAPDH.

*Construction of lncRNA PAX8-AS1:28 and MYC expression vector and transfection*. Cloning primers for PAX8-AS1:28 were 5'-CGTGTCGACCTCATTCATTTGTTC-3' (forward) and 5'-CGAAAGCTTAAAAAACTGACACATGC-3' (reverse). Cloning primers for MYC were 5'-CGAAAGCT TGCCACCATGCTCGGAAGGACTATCCTGCTGCCAA-3' (forward) and 5'-CGTGGATCCGGCGCTCCAAGACGT TGTGTGTTCG-3' (reverse). PCR amplified fragments with the U6 promoter were inserted into the pcDNA3.1 vector. IHH-4 cells were collected during the logarithmic growth phase, and were seeded in 6-well plates with $4.5x10^5$ cells per well. Cells were cultured in an incubator (37˚C, 5% $CO_2$). Five micrograms of PAX8-AS1:28 and MYC expression plasmid was diluted in 250 $\mu$l of serum-free medium and transfection was performed using Invitrogen™ Lipofectamine 2000 transfection reagent (cat. no. 11668-019, Thermo Fisher Scientific). Medium was replaced 4-6 h after transfection. After incubation for another 48 h, the cells were collected for subsequent experiments. Expression of PAX8-AS1:28 and MYC was detected after transfection to confirm the success of transfection.

*siRNA transfection*. MYC siRNA (cat. no. AM16708) and control siRNA (cat. no. AM4611) were purchased from Applied Biosystems/ThermoFisherScientific. The PAX8-AS1:28 siRNA target sequence was GAGAGGTCATTATGTGAAGGCT. siRNA (final concentration, 50 nM) was transfected into IHH-4 cells using Lipofectamine 2000 transfection reagent (cat. no. 11668-019, Thermo Fisher Scientific) according to the manufacturer's instructions. Medium was replaced 4-6 h after transfection. After incubation for another 48 h, the cells were collected for subsequent experiments.

*Cell proliferation assay*. Cell counting kit (CCK-8; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to measure cell proliferation ability according to the manufacturer's instructions. Briefly, 100 $\mu$l of cell suspension containing $5x10^3$ cells was transferred to each well of 96-well plates. Cells were cultured and CCK-8 solution (10 $\mu$l) was added into each well 24, 48 and 72 h later. After incubation at 37˚C for another 4 h, optical density (OD) values at 450 nm were measured using a microplate reader (Bio-Rad, USA).

*Western blot assay*. Total protein was extracted using cell lysis buffer (P0013K, Beyotime Institute of Biotechnology, Haimen, China), and the concentration was determined by BCA assay. Then, 20 $\mu$g of protein from each sample was subjected to 10% SDS-PAGE gel electrophoresis, followed by transmembrane to PVDF. Membranes were then blocked with 5% skimmed milk at room temperature for 1 h. After washing with TBST, membranes were then incubated with primary antibodies including rabbit anti-PAX8 (dilution 1:2,000, cat. no. ab53490, Abcam), rabbit anti-c-Myc (dilution 1:2,000, cat. no. ab32072, Abcam) and rabbit anti-β-actin (dilution 1:2,000, cat. no. ab8227, Abcam) overnight at 4˚C. After washing with PBS, the membranes were further incubated with anti-rabbit IgG-HRP secondary antibody (dilution 1:1,000, cat. no. MBS435036, MyBioSource) at room temperature for 2 h. Then the membranes were washed again with TBST, signals were detected using ECL (Sigma-Aldrich; Merck KGaA) method. Relative expression level of each protein was normalized to endogenous control β-actin using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

*Statistical analysis*. Statistical analyses were performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Normal distribution data were recorded as mean ± SD), and comparisons between two groups were performed by t-test. Correlation between the expression levels of AX8-AS1:28 and PAX8 mRNAs was analyzed by Spearman's rank correlation coefficient. $P<0.05$ was considered to be statistically significant.

## Results

*Expression of PAX8-AS1:28 and PAX8 mRNAs in PTC tissues and adjacent normal tissues*. qRT-PCR was performed to detect the expression of PAX8-AS1:28 and PAX8 mRNAs in PTC tissues and adjacent normal tissues. Compared with adjacent normal tissues, expression levels of PAX8-AS1:28 (Fig. 1A) and PAX8 (Fig. 1B) mRNAs were significantly reduced in PTC tissues ($P<0.01$). In addition, the expression level of PAX8-AS1:28 was positively correlated with expression of PAX8 (Fig. 1C,

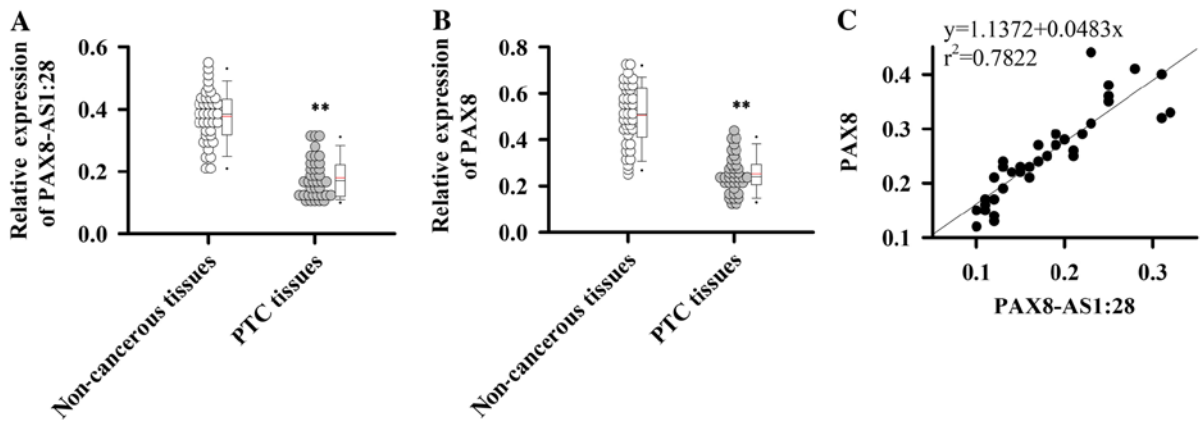


Figure 1. Expression of PAX8-AS1:28 and PAX8 mRNA in PTC and adjacent normal tissues. (A) Expression of PAX8-AS1:28 mRNA in PTC and adjacent normal tissues. GAPDH was used as an endogenous control. (B) Expression of PAX8 mRNA in PTC and adjacent normal tissues. GAPDH was used as an endogenous control. (C) Correlation between the expression of PAX8-AS1:28 and PAX8 mRNA. ** P<0.01, compared with the control group.

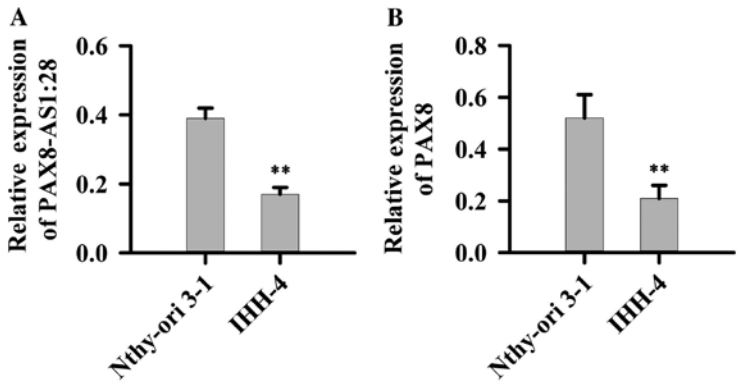


Figure 2. Expression of PAX8-AS1:28 and PAX8 in normal cell line and PTC cell line. (A) Expression of PAX8-AS1:28 in normal cell line Nthy-ori 3-1 and PTC cell line, IHH-4. GAPDH was used as an endogenous control. (B) Expression of PAX8 in normal cell line and PTC cell line. GAPDH was used as an endogenous control. ** P<0.01, compared with the control group.

$r^2$=0.7822). These data suggest that PAX8-AS1:28 and PAX8 are downregulated in PTC.

*Expression of PAX8-AS1:28 and PAX8 in normal and PTC cells*. Expression of PAX8-AS1:28 and PAX8 in normal cell line Nthy-ori 3-1 as well as PTC cell line IHH-4 was detected. Compared with normal cell line Nthy-ori 3-1, expression levels of PAX8-AS1:28 (Fig. 2A) and PAX8 mRNAs (Fig. 2B) were significantly reduced in the IHH-4 cells (P<0.01).

*Effect of PAX8-AS1:28 overexpression and silencing on PAX8 expression and IHH-4 cell growth*. After siRNA silencing, the expression level of PAX8-AS1 was significantly reduced (Fig. 3A), indicating the success of transfection. Compared with control cells, expression levels of PAX8 mRNA (Fig. 3A) and protein (Fig. 3B) were significantly reduced in IHH-4 cells following PAX8-AS1:28 siRNA silencing. In addition, PAX8-AS1:28 siRNA silencing significantly promoted the growth of IHH-4 cells (Fig. 3C).

Cells transfected with the PAX8-AS1:28 plasmid show an elevated expression level of PAX8-AS1:28 (Fig. 3D), indicating the successfully established PAX8-AS1 overexpression cell line. Compared with the control cells, expression levels of PAX8 mRNA (Fig. 3D) and protein (Fig. 3E) were significantly increased in IHH-4 cells with PAX8-AS1:28 overexpression. In addition, PAX8-AS1:28 overexpression significantly inhibited the growth of IHH-4 cells (Fig. 3F). These data suggest that PAX8-AS1:28 can positively regulate the expression of PAX8 to inhibit PTC.

*Expression of MYC in normal cell lines and PTC cell lines*. Expression of MYC in normal cell line Nthy-ori 3-1 and PTC cell line IHH-4 was detected. Compared with normal cell line Nthy-ori 3-1, expression levels of MYC were significantly increased in thr IHH-4 cells (P<0.01, Fig. 4).

*Effects of MYC overexpression and silencing on PAX8 and PAX8-AS1:28 expression and IHH-4 cell growth*. Cells transfected with the MYC plasmid showed elevated expression level of MYC (Fig. 5A and B), indicating the successfully established MYC overexpression cell line. Compared with the control cells, expression levels of PAX8-AS1:28 (Fig. 5A) and PAX8 (Fig. 5A and C) were significantly decreased in the IHH-4 cells with MYC overexpression. In addition MYC overexpression significantly promoted the growth of IHH-4 cells (Fig. 5D).

After siRNA silencing, expression level of MYC was significantly reduced (Fig. 5E and F), indicating the success of

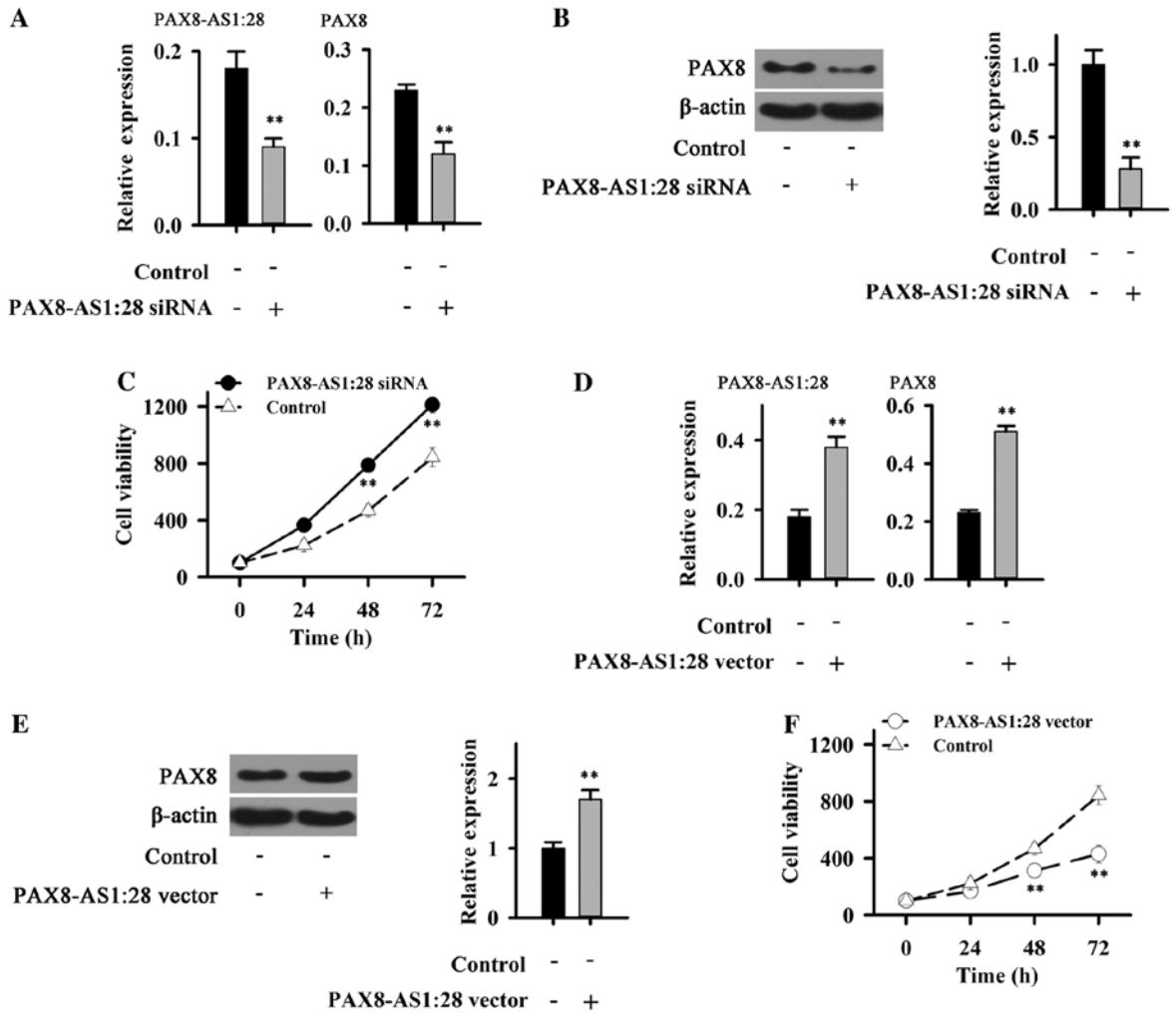


Figure 3. Effect of PAX8-AS1:28 overexpression and silencing on PAX8 expression and IHH-4 cell growth. (A) Effect of PAX8-AS1:28 siRNA silencing on PAX8-AS1:28 and PAX8 RNA expression. (B) Effect of PAX8-AS1:28 siRNA silencing on PAX8 protein expression. β-actin was used as an endogenous control. (C) Effect of PAX8-AS1:28 siRNA silencing on cell growth. (D) Effect of PAX8-AS1:28 overexpression on PAX8-AS1:28 and PAX8 RNA expression; (E) Effect of PAX8-AS1:28 overexpression on PAX8 protein expression. (F) Effect of PAX8-AS1:28 overexpression on cell growth. β-actin was used as a endogenous control. ** P<0.01, compared with the control group.

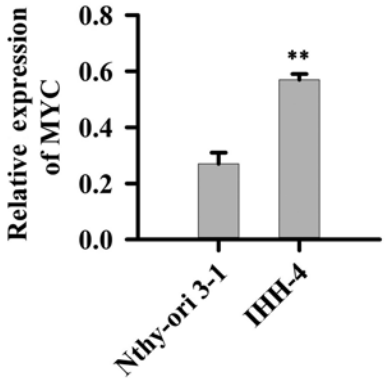


Figure 4. Expression of MYC in normal cell line Nthy-ori 3-1 and PTC cell line IHH-4. GAPDH was used as an endogenous control. ** P<0.01, compared with the control group.

transfection. Compared with the control cells, expression levels of PAX8-AS1:28 (Fig. 5E) and PAX8 protein (Fig. 5G) were significantly reduced in IHH-4 cells following MYC siRNA silencing. In addition, MYC siRNA silencing significantly inhibited the growth of IHH-4 cells (Fig. 5H). These data suggest that MYC can negatively regulate the expression of PAX8-AS1:28 and PAX8 to promote PTC.

## Discussion

The pathogenesis of PTC is still unclear. Therefore, understanding the molecular pathogenesis and mechanisms underlying PTC is still a 'hot research spot' regarding the treatment of PTC (12). Recent studies have identified multiple pathways that are related to the development and progression of PTC, such as the HIF-1α pathway (13), the thyroid-stimulating hormone receptor signaling pathway (14) and the WNT/β-catenin signaling pathway (15). Epigenetic and genetic alterations in those pathways, including altered gene copy-number, gene mutation and aberrant gene methylation play central roles in the pathogenesis of PTC (12). A variety of lnRNAs have also been proven to be involved in the development of PTC. lncRNA SLC6A9 was found to be downregulated in 131I-resistant PTC accompanied by the inhibition of PARP-1, and a high expression level of SLC6A9

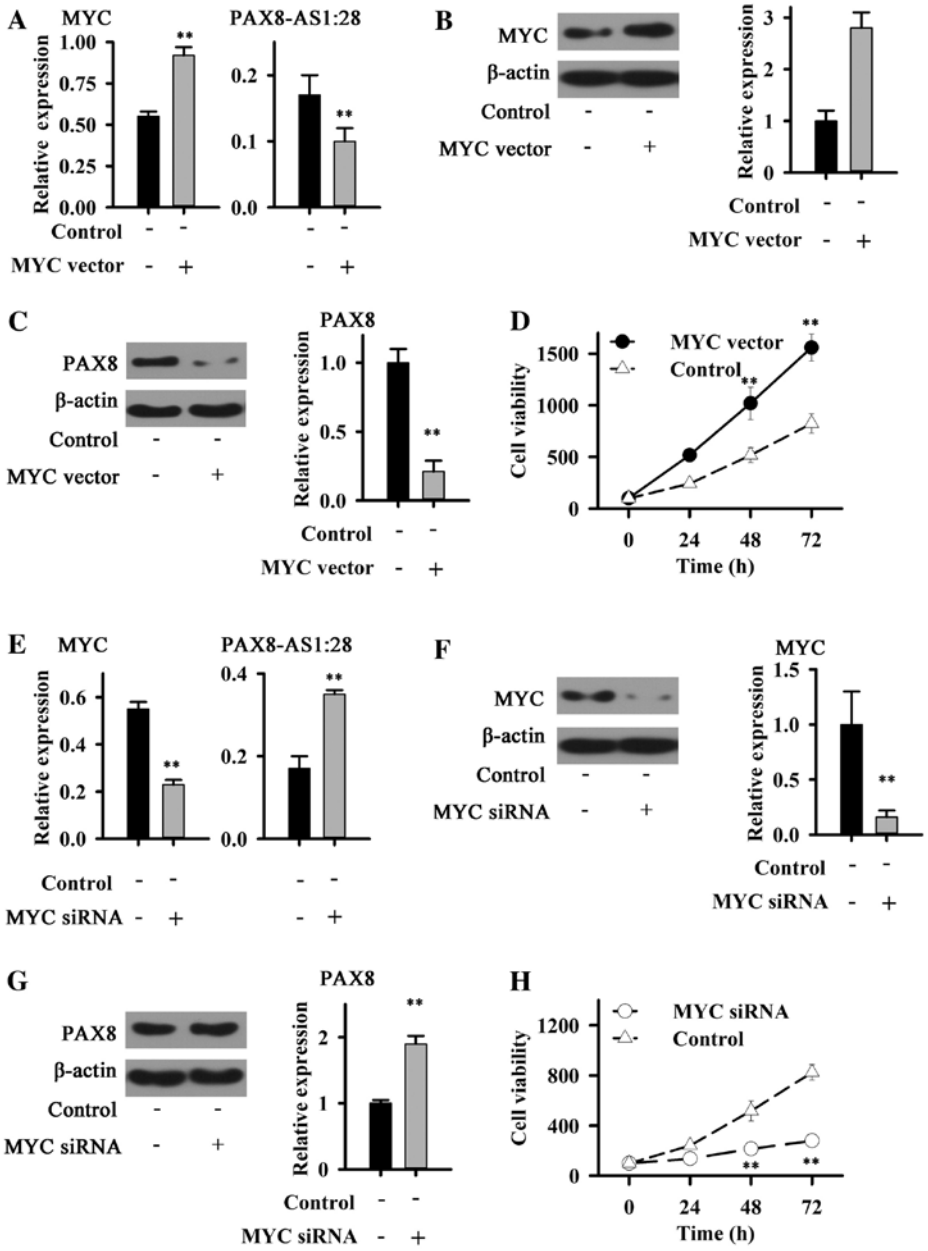


Figure 5. Effects of MYC overexpression and silencing on PAX8 and PAX8-AS1:28 expression and IHH-4 cell growth. (A) Effect of MYC overexpression on MYC mRNA and PAX8-AS1:28 RNA expression. (B) Effect of MYC overexpression on MYC protein expression. β-actin was used as an endogenous control. (C) Effect of MYC overexpression on PAX8 protein expression. β-actin was used as an endogenous control. (D) Effect of MYC overexpression on cell growth. (E) Effect of MYC silencing on MYC mRNA and PAX8-AS1:28 RNA expression. β-actin was used as an endogenous control. (F) Effect of MYC silencing on MYC protein expression. (G) Effect of MYC silencing on PAX8 protein expression. β-actin was used as an endogenous control. (H) Effect of MYC silencing on cell growth.** P<0.01, compared with the control group.

was found to be positively correlated with the overall survival rate and disease-free survival rate of PTC patients who received 131I therapy, indicating that SLC6A9 can potentially serve as a novel target for the treatment of 131I-resistant PTC (16). In addition to the direct effects on PTC, lncRNAs can also interact with key signal transduction pathways that are involved in the pathogenesis of PTC. In a recent study, lncRNA PTCSC was proven to significantly regulate the expression of SCAI and subsequently alter the activity of Wnt/β-catenin signal transduction, which in turn regulated the proliferation and migration of PTC cells (17). All of these previous studies suggest that lncRNA are key players in the pathogenesis of PTC.

lnc-PSD4-1:14, or lncRNA PAX8-AS1:28, is a newly discovered lncRNA. Based on our knowledge, the functionality of lncRNA PAX8-AS1:28 is still unknown. It has been reported that the expression level of lncRNA PAX8-AS1:28 is commonly decreased in patients with neck squamous cell carcinoma, and a higher expression level of lncRNA PAX8-AS1:28 is closely correlated with better survival outcome (18). lncRNA PAX8-AS1:28 expression is also downregulated in patients with PTC (11), and the reduced expression level of lncRNA PAX8-AS1:28 is closely correlated with the poor survival of these patients (19). Consistent with previous studies, in our study, the expression level of PAX8-AS1:28 was found to be lower in PTC tissues and PTC cells than that in adjacent

healthy tissues and a normal cell line. Our finding further confirmed the involvement of PAX8-AS1:28 in PTC.

Paired box gene 8, or PAX8, is a transcription factor that belongs to the paired box (PAX) family (20). Mutations in PAX8 have been proven to be related with various thyroid diseases including thyroid follicular carcinomas, thyroid dysgenesis and atypical follicular thyroid adenomas (20). lncRNA PAX8-AS1:28 overlaps with paired box 8 (PAX8) in an antisense orientation (18), indicating the possible interactions between PAX8 and lncRNA PAX8-AS1:28. In this study, PAX8 expression was also significantly downregulated in PTC tissues and PTC cells than that in adjacent healthy tissues and normal cells. In addition, lncRNA PAX8-AS1:28 expression was found to be positively correlated with the expression of PAX8. Moreover, lncRNA PAX8-AS1:28 silencing reduced the expression level of PAX8 and promoted PTC cell growth. In contrast, lncRNA PAX8-AS1:28 overexpression increased the expression level of PAX8 and inhibited PTC cell growth. Those data suggest that PAX8-AS1:28 may affect PTC cell growth by positively regulating the expression of PAX8.

As an oncogene, MYC is overexpressed in various tumor tissues, and MYC overexpression promotes the proliferation, migration and invasion of tumor cells (21). An increased expression level of MYC has also been detected in patients with PTC (22), indicating the involvement of MYC in this disease. Consistent with previous studies, in our study, the expression level of MYC in PTC cells was found to be significantly higher than that in a normal cell line. In a recent study, protein levels of MYC and PAX8 were found to be inversely correlated with each other in thyroid tumors (23), indicating the opposite roles of those 2 proteins in thyroid cancer. In our study, MYC silencing increased expression levels of PAX8-AS1:28 as well as PAX8 and inhibited tumor cell growth, while MYC overexpression decreased expression levels of PAX8-AS1:28 as well as PAX8 and promoted tumor cell growth. MYC is multifunctional and nuclear phosphoprotein that is involved in the expression regulation of a large set of genes. The downregulation of PAX8-AS1:28 and PAX8 may be caused by the direct role of MYC or its downstream targets. All these data suggest that MYC can promote PTC, and this function may be correlated with the downregulation of expression of PAX8-AS1:28 and PAX8. It is interesting that antisense PAX8-AS1:28 elevated the expression of PAX8. The possible explanation is that PAX8-AS1:28 may be involved in the post-transcriptional process of PAX8 transcripts, which has been observed in a recent study (24).

In conclusion, the expression level of PAX8-AS1:28 and PAX8 were lower in PTC tumor tissue and PTC cell lines than that in healthy tissue and normal cell lines, while the expression level of MYC was higher in PTC cell lines than that in normal cell lines. PAX8-AS1:28 can positively regulate PAX8 expression and inhibit PTC tumor cell growth. In contrast, MYC can negatively regulate the expression of PAX8-AS1:28 as well as PAX8 and promote tumor cell growth. Therefore, PAX8-AS1:28 and PAX8 may serve as biomarkers for the diagnosis of PTC. They may also serve as targets for the treatment of this disease. The present study was still limited by the small sample size. Further studies with a larger sample size are needed to further confirm the conclusions in the present study.

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**Availability of data and materials**

The datasets used during the present study are available from the corresponding author upon reasonable request.

**Authors' contributions**

YZ and JC conceived and designed the study. YZ and FL performed the experiments. JC wrote the paper. YZ and FL reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Ethics approval and consent to participate**

The Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) approved this study and all patients provided informed consent.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors state that they have no competing interests.

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