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Original Article

Proto-oncogene RTL4 promotes tumorigenesis and invasiveness of papillary thyroid cancer

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Abstract: Background: Although the prognosis of papillary thyroid carcinoma (PTC) is good, its widespread prevalence still degrades the quality of life of tens of thousands of patients. PTC can even be life-threatening as a result of its aggressiveness and metastasis. Methods: Using complete transcriptome sequence analysis, cutting-edge research has revealed many tumor-associated genes. These related genes help us better understand the tumorigenesis and progression of PTC. We discovered that retrotransposon Gag like 4 (RTL4) is a novel potential PTC-associated gene. By Quantitative real-time polymerase chain reaction (qRT-PCR), we observed an obvious upregulation of RTL4 in PTC tissue. And, we validated the expression characteristics of RTL4 using data from the Cancer Genome Atlas (TCGA). Furthermore, we down-regulated RTL4 expression levels in relevant cell lines and studied the biological function of the RTL4 line in PTC by cell proliferation, colony formation, migration and invasion assays. Results: In the present study, high expression of RTL4 suggested lymph node metastasis (P = 0.028) and was associated with the pathological type (P = 0.001). RTL4 had the validity of distinguishing PTC tissues and normal tissues showed an AUC of 87.53% for the TCGA data set. The downregulation of RTL4 in the PTC cell lines distinctly inhibited cell colony formation, proliferation, migration, and invasion. Conclusions: The result revealed RTL4 is closely related to the occurrence and development of PTC. RTL4 may participate in the HOTAIR-miR-206-ZCCHC16 ceRNA regulatory network and be regulated and play a role in the ceRNA regulatory network. It may be used as a target or indicator for the treatment and prognosis of PTC.

Keywords: Papillary thyroid carcinoma, gene, retrotransposon Gag like 4

Introduction

Thyroid carcinoma (TC) is the most common endocrine system tumor [1]. In recent years, its global incidence has continued to rise [2], mainly as a result of the growing use of many effective inspection methods. Papillary thyroid carcinoma (PTC) belongs to differentiated thyroid carcinoma and is the most common type in clinical practice, followed by follicular thyroid carcinoma (FTC). Most of the prognosis is good, but some patients have a high recurrence rate and poor prognosis. PTC has the characteristics of slow tumor growth, low degree of deterioration, and long latency [3]. However, anaplastic thyroid cancer (ATC) is a very aggressive solid tumor [4]. Therefore, Doctors face a challenge is how to balance the treatment strategy so that patients with benign thyroid nodules or lower risk disease are not over treated. In the meantime, they need to identify the patients at high risk or more advanced, who need a more aggressive treatment plan [5]. Understanding the molecular mechanisms of the development and progression of TC is an important basis for providing accurate diagnosis and personalized treatment [6]. Follow with the significant developments of next-generation sequencing (NGS), several studies have found many genetic changes related to TC. Several characteristic molecular markers have been identified, such as chromosomal rearrangements (RET/PTC1, RET/PTC3, and PAX8/PPARG) and point mutations of proto-oncogenes (BRAF, NRAS, HRAS, and KRAS), which can be used to distinguish histological subtypes [5]. By detecting mutations in susceptibility genes, the early diagnosis accuracy of thyroid malignant tumors is significantly improved, and clinical treatment is guided [7].

The molecular mechanism of PTC has not been fully elucidated, although notable progress in

the genetic study had been done. In the previous work, the study collected 19 pairs of primary thyroid cancer tissues and matched normal thyroid samples, and performed the whole transcriptome sequencing matched normal thyroid samples [8]. Then, using bioinformatics analysis methods, we found that retrotransposon Gag like 4 (RTL4) significantly up-regulated in PTC. Previous studies suggest that overexpressed genes in PTC may be potential markers for molecular diagnostics [9]. Defining clinically useful predictors of high-risk factors is the key to the diagnosis and treatment of PTC. RTL4 is one of these genes.

RTL4, also named Zinc-finger CCHC domain-containing 16 (SIRH11/ZCCHC16), is an X-linked gene. The function of RTL4 is to encode a CCHC-type zinc finger protein. RTL4 and LTR retrotransposon Gag protein show high identity in sequence [10]. Previous studies have found that RTL4 expression can be detected in the brain, kidney, liver, and thyroid of adult mice. The deletion of RTL4 may result in cognitive-related abnormal behavior in mice, including attention, impulsivity, and working memory, which may be related to the blue spot-norepinephrine (LC-NA) system [10, 11]. RTL4 may also function as a non-coding RNA. In our studies, transcriptome sequence analysis revealed that the RTL4 gene is closely related to PTC. Our study sought to understand the link between RTL4 expression and clinical features of PTC and to study the biological effects of RTL4.

Materials and methods

Patient tissue samples

Between 2016 and 2018, PTC samples and matched normal thyroid samples were obtained from 40 PTC patients at the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China). Matched normal thyroid samples were gotten from a distance of 3-5 cm from the edge of the tumor on the same patient. All samples were histologically analyzed by two pathologists at least. All samples were immediately frozen in liquid nitrogen. We obtain the RTL4 Reads Per Kilobase per Million reads (RPKM) expression value from The Cancer Genome Atlas (TCGA) portal (https://cancergenome.nih.gov/). In total, 507 PTC sequence data with complete clinical features and 58 pairs of thyroid cancer with matched normal samples were selected.

Ethics statement

This study was in line with the Helsinki Declaration and government policies and was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. All patients who participated in the study signed informed consent.

Main reagents and instruments

Fetal bovine serum (FBS), RPMI 1640, Trizol® reagent, RNAiMAX and ABI 7500 Real-time PCR System was purchased from Invitrogen (Carlsbad, CA, USA). ReverTra Ace® qPCR RT Kit and THUNDERBIRD SYBR qPCR Mix were purchased from Toyobo (Osaka, Japan). Small interfering RNA (siRNA) for RTL4 was purchased from Shanghai Gene Pharma (Shanghai, China). Paraformaldehyde (PFA) was purchased from Merck Millipore (Darmstadt, Germany). Transwell chambers with membranes were purchased from Corning Inc. (Corning, NY, USA). The membranes were uncoated for the migration experiments and were coated with 25 μg Matrigel® (BD Biosciences, Franklin Lakes, NJ, USA) for the invasion experiments. ImageJ 1.5 software was obtained from the National Institutes of Health (Bethesda, MD, USA). The SPSS 23.0 software was obtained from SPSS Inc. (Chicago, IL, USA).

Cell lines and cell culture

The human TC cell line (BCPAP and TPC1) was purchased from the Shanghai Stem Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 medium containing 10% FBS, 1% NEAA, 1% sodium pyruvate, were conserved in a humidified incubator (95% air/5% CO_2) at 37°C.

RNA extraction and qRT-PCR (quantitative real-time PCR)

Briefly, total RNA was extracted using Trizol® reagent from the samples or cell lines in the light of the standard experimental procedure. In the light of standard experimental procedure (20 μl reaction: 14 μl RNA + 4 μl 5xRT Buffer + 1 μl Primer Mix + 1 μl EnzymeMix, step1: 16°C for 5 min, step2: 42°C for 30 min, step3: 98°C

for 5 min), cDNA was synthesized by reverse transcription. cDNA samples were stored at -80°C. To detect RNA expression, Real-time PCR was performed in the light of standard experimental procedure (10 μl reaction: 0.5 μl cDNA + 3.5 μl RNA-free water + 0.4 μl forward primer + 0.4 μl reverse primer + 0.2 μl ROX + 5 μl Thunderbird SYBR qPCR Mix, the concentration of primer was 0.2 μM, step1: 95°C for 2 min, step2: 95°C for 15 sec, step3: 60°C for 60 sec, repeat step2 and step3 for 40 cycles, final step: 72°C for 5 min). Using GAPDH as an internal reference, The relative expression of the gene was expressed by the $2^{-\Delta\Delta CT}$ value [12]. The primer sequences for PCR are as follows: RTL4, forward 5'-CTACGATCTACCTTCAGCATA-3' and reverse 5'-ACAGTTCCAAGCAGTATTCA-3'; GAPDH, forward 5'-GGTCGGAGTCAACGGATTTG-3' and reverse 5'-ATG AGCCCCAGCCTTCTCCAT-3'.

Transfection

The BCPAP (1.2×10^5 cells) and TPC1 (6×10^4 cells) cell lines were cultured in 6-well plates 24 h earlier than transfection. RTL4 was knocked down in TPC1 cell lines using 10 μL siRNA (20 μM) and 4 μL RNAiMAX for 48 h. Similarly, RTL4 was knocked down in BCPAP cell lines using 5 μL siRNA (20 μM) and 2 μL RNAiMAX for 48 h. Then, the cells were selected for further experiments, and RT-qPCR analysis testified the efficiency of knockdown. The sequences of siRNA as follows: siRNA1, forward 5'-GCAGGUAGAGCCUUCCUUUTT-3' and reverse 5'-AAAGGAAGGCUCUACCUGCTT-3'; siRNA2, forward 5'-CCAACACCAAGCCCUCUUUTT-3' and reverse 5'-AAAGAGGGCUUGGUGUUGGTT-3'. Each experiment was repeated three times at least.

Colony formation assay

The transfected and control cells (1×10^3 cells for TPC1 and BCPAP) were seeded in each well of six-well plates. They were cultured in the growth medium containing 10% FBS at 37°C with 5% CO_2, and the growth medium was changed every 2 d. After 7 d, their colonies were visible to the naked eyes. Finally, (PFA) the cells were fixed using 4% paraformaldehyde for 30 min and stained with 0.01% crystal violet for 30 min. Finally, the numbers of the colonies were photographed and counted. Each experiment was repeated three times at least.

CCK-8 proliferation assay

About 1000 cells were seeded in each well of 96-well plates. 10 uL CCK-8 solution was added to each well after cells adhered. Then, the 96-well plates were incubated for 96 h at 37°C. Using a microplate reader, the absorbance at 450 nm was measured at 24 h, 48 h, 72 h, 96 h. Each experiment was repeated three times at least.

Migration and invasion assays

We used trypsin to trypsinize cells and collected cells using medium containing 10% FBS. In the lower chamber, we added a 600 ml medium containing 10% FBS. Into the upper chamber, transfected cells or control cells (1000 cells, ~300 ml) were plated. Then, the cells were incubated for 22 h. Finally, the Cells on the bottom of the membrane were fixed for 20 min and stained for 20 min. Matrigel invasion and migration assay were similar. Finally, the cells that had migrated through the membrane were photographed and counted. Photographs were captured while counting cells using the optical microscope (×20). The ability of migration and invasion was measured by counting results. Each experiment was repeated three times at least.

Statistical analysis

The SPSS 23.0 software was used for all statistical analyses; Normal distribution data are presented as mean ± standard error of the mean; The differences between groups were tested using Student's t-test (two-tailed); If $P<0.05$, the difference was statistically significant.

Result

High expression of RTL4 in PTC tumor tissues

We analyzed the expression level of RTL4 in TC with matched normal thyroid samples in TCGA (**Figure 1A**, $P<0.05$). We found that RTL4 was significantly increased in TC tissues compared with matched normal thyroid samples. Using RT-qPCR, we evaluated the expression level of RTL4 among 30 pairs of PTC samples and matched normal thyroid samples obtained during clinical operations. The results showed that, compared with the normal tissues, the expression level of RTL4 was markedly overexpressed

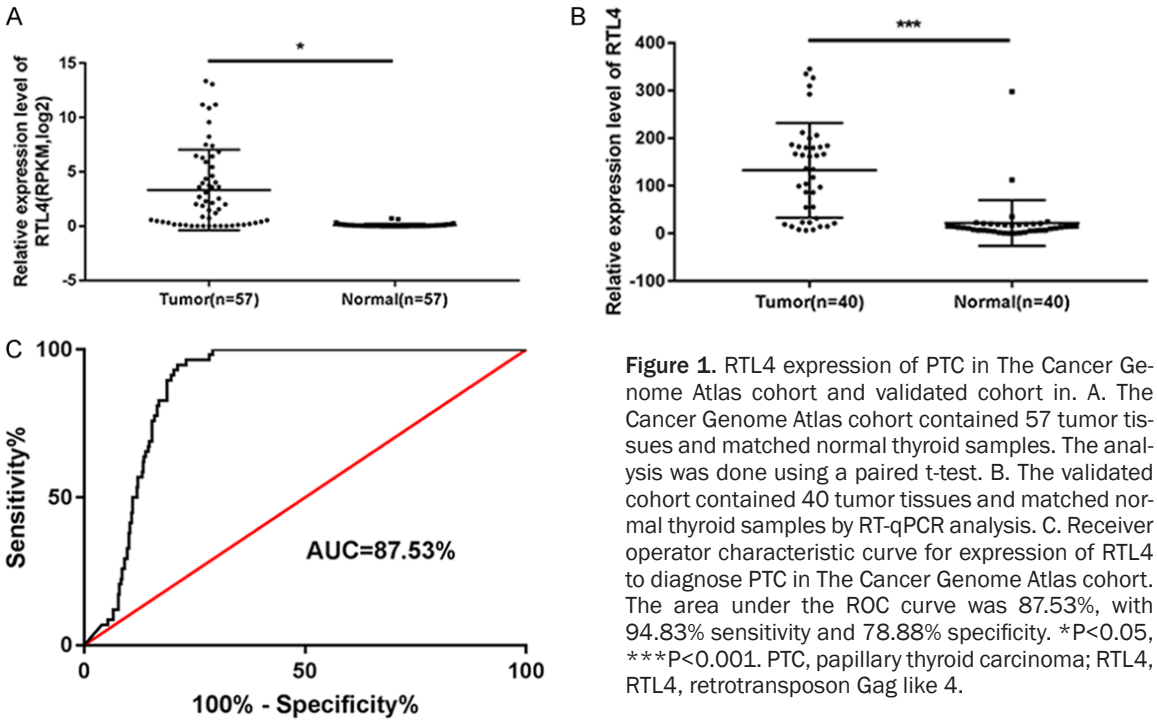

Figure 1. RTL4 expression of PTC in The Cancer Genome Atlas cohort and validated cohort in. A. The Cancer Genome Atlas cohort contained 57 tumor tissues and matched normal thyroid samples. The analysis was done using a paired t-test. B. The validated cohort contained 40 tumor tissues and matched normal thyroid samples by RT-qPCR analysis. C. Receiver operator characteristic curve for expression of RTL4 to diagnose PTC in The Cancer Genome Atlas cohort. The area under the ROC curve was 87.53%, with 94.83% sensitivity and 78.88% specificity. *P<0.05, ***P<0.001. PTC, papillary thyroid carcinoma; RTL4, RTL4, retrotransposon Gag like 4.

in the tumor samples, (**Figure 1B**, P<0.001). It was consistent with the results mentioned above. Furthermore, to evaluate the latent diagnostic value of RTL4, ROC analysis was done. Using RTL4 to identify TC tissues and normal thyroid tissues, the AUC (area under the curve) value is 0.8753 and the sensitivity/specificity is 94.83/78.88% (**Figure 1C**) for the TCGA data sets.

Association between RTL4 and clinical features

According to the median value of RTL4, the PTC patient group was divided into a high expression group and a low expression group. In TCGA, RTL4 was markedly associated with lymph node metastasis (P = 0.028) and Histological type (P = 0.001), as shown in **Table 1**. But, RTL4 was There was no statistical difference between RTL4 and other factors, such as age (P = 0.200), gender (P = 0.054), tumor size (P = 0.285), metastasis (P = 0.967), or Clinical stage (P = 0.508).

RTL4 increases the risk of lymph node metastasis of PTC

Univariate logistic regression analysis revealed that the marked variables for lymph node metastasis were RTL4 (OR 1.533, 95% CI 1.058-2.222, P = 0.024), age (OR 0.620, 95% CI 0.427-0.899, P = 0.012), gender (OR 0.645, 95% CI 0.425-0.987, P = 0.039), clinical-stage (OR 3.493, 95% CI 2.316-5.268, P<0.001), histological type (OR 0.420, 95% CI 0.272-0.648, P<0.001) and tumor size (OR 2.525, 95% CI 1.652-3.858, P<0.001), as shown in **Table 2**. To avoid possible multi-colinear interference between independent variables, we further carried out the multivariate logic analysis as shown in **Table 3**. RTL4 (OR 1.723, 95% CI 1.107-2.682, P = 0.016), age (OR 0.030, 95% CI 0.009-0.098, P<0.001), clinical-stage (OR 61.804, 95% CI 18.145-210.508, P≤0.001) and histological type (OR 0.363, 95% CI 0.220-0.599, P<0.001) were significantly related to lymph node metastasis, and lymph node metastasis status was not correlated with gender and tumor size. So, RTL4 increased the risk of lymph node metastasis in patients with PTC.

RTL4 promotes PTC cell lines proliferation in vitro

Assessed RTL4 in several normal thyroid cell lines and PTC cell lines by qRT-PCR, we found that RTL4 had a higher level in PTC cell lines than normal thyroid cell line HTORI3 (**Figure 2A**). RTL4 was commonly up-regulated in PTC,

Table 1. Association between the expression of RTL4 and clinicopathological features in The Cancer Genome Atlas cohort

Clinicopathological features	Low RTL4 expression (n = 255), n (%)	High RTL4 expression (n = 252), n (%)	*P*-value
Age (years)			0.200
Mean ± SD	49.17±16.04	45.34±15.34	
≤45	142	126	
>45	113	126	
Gender			0.054
Male	78	58	
Female	177	194	
Primary tumor size (T)			0.285
T1	67	77	
T2/T3/T4	187	174	
Lymph node metastasis			0.028*
NO	127	104	
YES	101	125	
Metastasis			0.967
NO	139	144	
YES	5	4	
Clinical stage			0.508
I+II	166	171	
III+IV	88	80	
Histological type			0.001*
Classical	164	195	
Other	91	57	

RTL4, retrotransposon Gag like 4; SD, standard deviation. *P<0.05.

Table 2. Univariate logistic regression analysis for the risk of lymph node metastasis

Factor	OR	95% CI	*P*-value
RTL4 expression (low vs. high)	1.533	1.058-2.222	0.024
Age, years (≤45 vs. >45)	0.620	0.427-0.899	0.012
Gender (male vs. female)	0.645	0.425-0.987	0.039
T (T1 vs. T2/T3/T4)	2.525	1.652-3.858	0.000
Metastasis (NO vs. YES)	1.510	0.332-6.876	0.594
Clinical stage (I+II vs. III+IV)	3.493	2.316-5.268	0.000
Histological type (Classical vs. Other)	0.420	0.272-0.648	0.000

RTL4, retrotransposon Gag like 4.

so we hypothesized that it is important in tumorigenesis and progression. For confirming this hypothesis, the present research chose impactful siRNA to effectively knock down RTL4 in the cell lines, and we used RT-qPCR to assess RTL4 after transfection, and the results showed a high transfection efficiency (**Figure 2A** and **2B**). Colony formation assays and Cell proliferation assays were then done. The outcomes showed that compared with the contrast group, the down-regulation of RTL4 markedly inhibited thyroid cell line colony formation (**Figure 3C** and **3D**, P<0.01) and proliferation (**Figure 3A** and **3B**, P<0.0001), compared with the contrast group.

RTL4 promotes PTC cell lines migration and invasion

As mentioned above, RTL4 was associated with lymph node metastasis, so we investigated the effect of RTL4 on the metastasis ability of TC cells using migration and invasion assays. The data exposed that the RTL4 inhibitor groups exhibited markedly reduced migration compared with the contrast group in the two cell lines (**Figure 4A** and **4C**, P<0.001). The Transwell invasion assays also exposed that down-regulated RTL4 markedly inhibited the invasion ability of the PTC cell lines (**Figure 4B** and **4D**, P< 0.001). Combined clinical analysis results, these experimentations mentioned above indicated that RTL4 is a risk factor for metastasis in PTC.

Discussion

During the past few decades, the incidence of TC has increased steadily [13]. PTC is the most common type of thyroid malignancy, constituting more than 95% of all cases of thyroid malignancy [14]. With a 10-year survival rate of >95%, PTC is well known for its excellent prognosis. However, about 10-15% of PTC patients develop recurrence after the initial surgery [15, 16], and even about 35% of them die due to

Table 3. Multivariate logistic regression analysis for risk of lymph node metastasis

Factor	OR	95% CI	*P*-value
RTL4 expression (low vs. high)	1.723	1.107-2.682	0.016
Age, years (≤45 vs. >45)	0.030	0.009-0.098	0.000
Gender (male vs. female)	0.670	0.405-1.110	0.120
T (T1 vs. T2/T3/T4)	1.463	0.861-2.484	0.160
Clinical stage (I+II vs. III+IV)	61.804	18.145-210.508	0.000
Histological type (Classical vs. Other)	0.363	0.220-0.599	0.000

RTL4, retrotransposon Gag like 4.

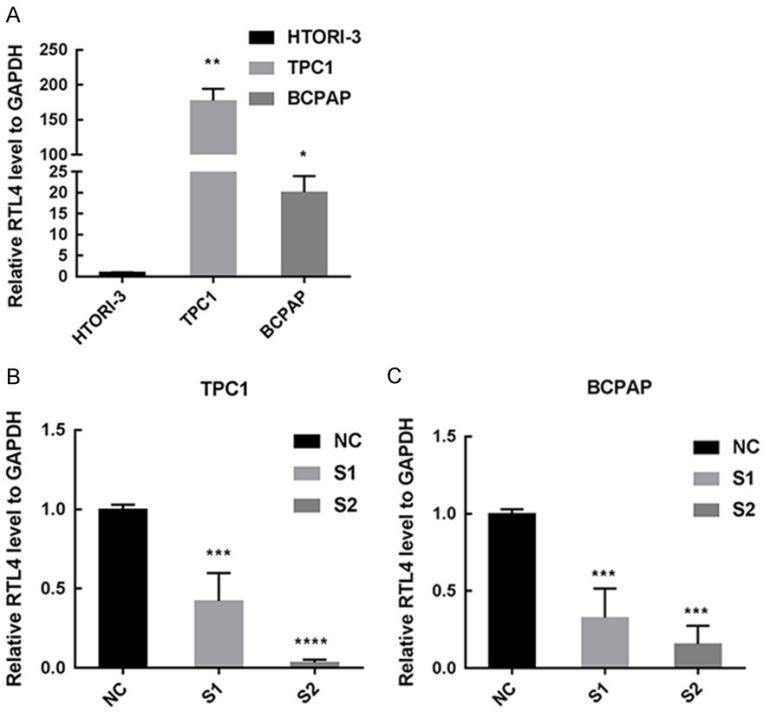

Figure 2. RTL4 expression in PTC cells and after transfected with small interfering RNA (siRNA) by RT-qPCR. A. The relative expression of RTL4 in PTC cells compared with HTORI-3 by RT-qPCR. Compared to HTORI-3, TPC1 and BCPAP exhibited a significantly high expression of RTL4 expression. B, C. After transfected with siRNA, the expression level of RTL4 in TPC1 and BCPAP cells, compared with the NC group. *P<0.05; **P<0.01; ***P<0.001. A logarithmic scale of 2-ΔΔCt is used to represent the fold change in quantitative real-time PCR detection; NC, normal control; siRNA, small interfering RNA; S1, siRNA1; S2, siRNA2.

PTC. The poor prognosis is mainly caused by tumor invasion and metastasis [17]. PTC is highly inclined to lymph node metastasis. On the initial presentation, over one-third of patients have clinically detectable lymph node involvement [18, 19]. Although there is no detectable lymph nodal disease during the preoperative examination, many patients have micro-metastatic lymph node disease in postoperative pathological examination [20]. More than a few studies have acknowledged that lymph node metastasis is a poor prognostic factor, which can increase the risk of local recurrence [21]. If the tumor has lymph node metastasis, the current treatment is lymph node dissection. There is currently no reliable means to accurately determine the correct lymph node metastasis before surgery. To accurately guide whether lymph node dissection is needed, it is significant to identify a lot of genes associated with PTC as molecular markers, which can forecast lymph node metastasis and prognosis of PTC [22].

Although researchers have done extensive research on thyroid cancer in the past few decades, no known oncogene drivers or epigenetic changes have been found in about 4% of PTC cases [23]. In our researches, RTL4 had a significantly high expression in the analysis of the sequencing dataset, so RTL4 might be a significant PTC-associated gene. Also, it was confirmed in TCGA datasets and verified in the organization by RT-qPCR. High expression of RTL4 suggests that RTL4 plays an important role in the occurrence and development of PTC.

We first proposed its role as a pro-oncogene in TC. It is also possible that RTL4 functions as a non-coding RNA [11]. RTL4 has also been identified as an independent risk factor for lymph node metastasis in patients with PTC. The discovery of the role of RTL4 in thyroid cancer is conducive to a deeper understanding of the mechanisms associated with TC. RTL4 may help improve some existing PTC prognostic models and be used as targets.

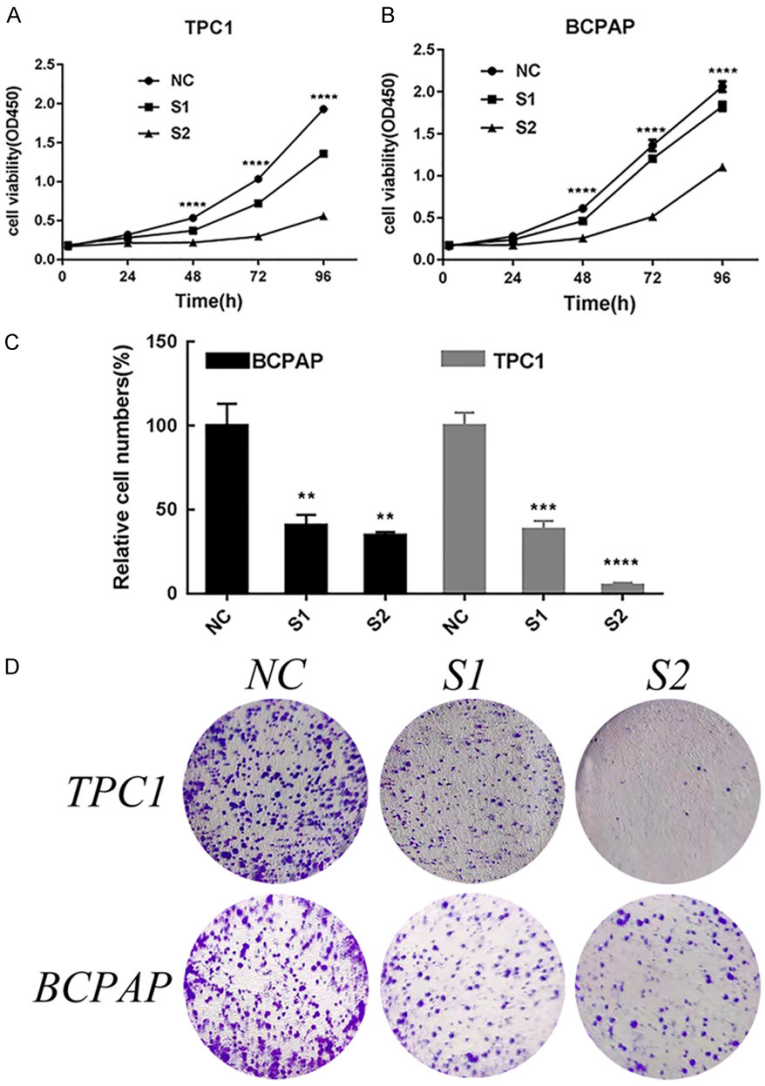

Figure 3. Downregulated expression of RTL4 for cell proliferation assay and colony formation assay in PTC. In the cell proliferation assay, compared with the NC group, proliferation was significantly suppressed in (A) TPC1 and (B) BCPAP cells transfected with siRNA; Cell proliferation was measured using CCK-8. (C) Relative quantification of the colony numbers in the (D) colony formation assay: in which TPC1 and BCPAP cells transfected with siRNA were cultured in six-well plates for 7d at the appropriate density. **P<0.01; ***P<0.001, ****P<0.0001. RTL4, RTL4, retrotransposon Gag like 4.

Pandolfi et al proposed the competitive endogenous RNA (ceRNA) hypothesis in 2011, that is, specific RNAs (including lncRNA, mRNA, pseudogenes, and circular RNAs) can be incorporated into miRNAs through a common miRNA response element (MRE) to destroy miRNA activity and subsequently up-regulate target RNA expression [24]. This hypothesis introduces a new RNA-RNA crosstalk theory that widely regulates gene expression. Since then, the frontiers of revealing ceRNA mechanisms have become increasingly hot. At present, researchers have constructed lncRNA-miRNA-mRNA networks in many malignant tumors that may cause tumorigenesis and progression.

Recent studies have initially revealed several ceRNA regulatory interactions and corresponding mechanisms in thyroid cancer. LncRNA H19 up-regulates the target gene YES1 by spongy miR-17-5p, thus proving it as ceRNA. Prove that ceRNA crosstalk including H19, miR-17-5p and YES1 may exist in the pathogenesis of thyroid cancer [25]. Based on data analysis, some studies reported that the lncRNA-miRNA-mRNA interaction of HOTAIR-miR-206-KLK-10 (or HOTAIR-miR-206-DACH2, -HTR1D, ZCCHC16, or -TRPC5) exists in the ceRNA regulatory network in PTC. Decreased expression of miR-18b may significantly up-regulate the five genes mentioned above [26]. Therefore, RTL4 is likely to participate in the regulation and function of the HOTAIR-miR-206-ZCCHC-16 ceRNA regulatory network.

As with others, there are some shortcomings in our research. First, our research lacks animal experiments. Second, the number of clinical cases is limited, and more cases can provide more accurate results. Third, the mechanism by which RTL4 works needs to be further clarified.

In our study, we found that the association between RTL4 and lymph node metastasis, and the function of RTL4 in vitro. We found that RTL4 was distinctly associated with lymph node metastasis and RTL4 caused increased tumorigenesis and metastasis. These results

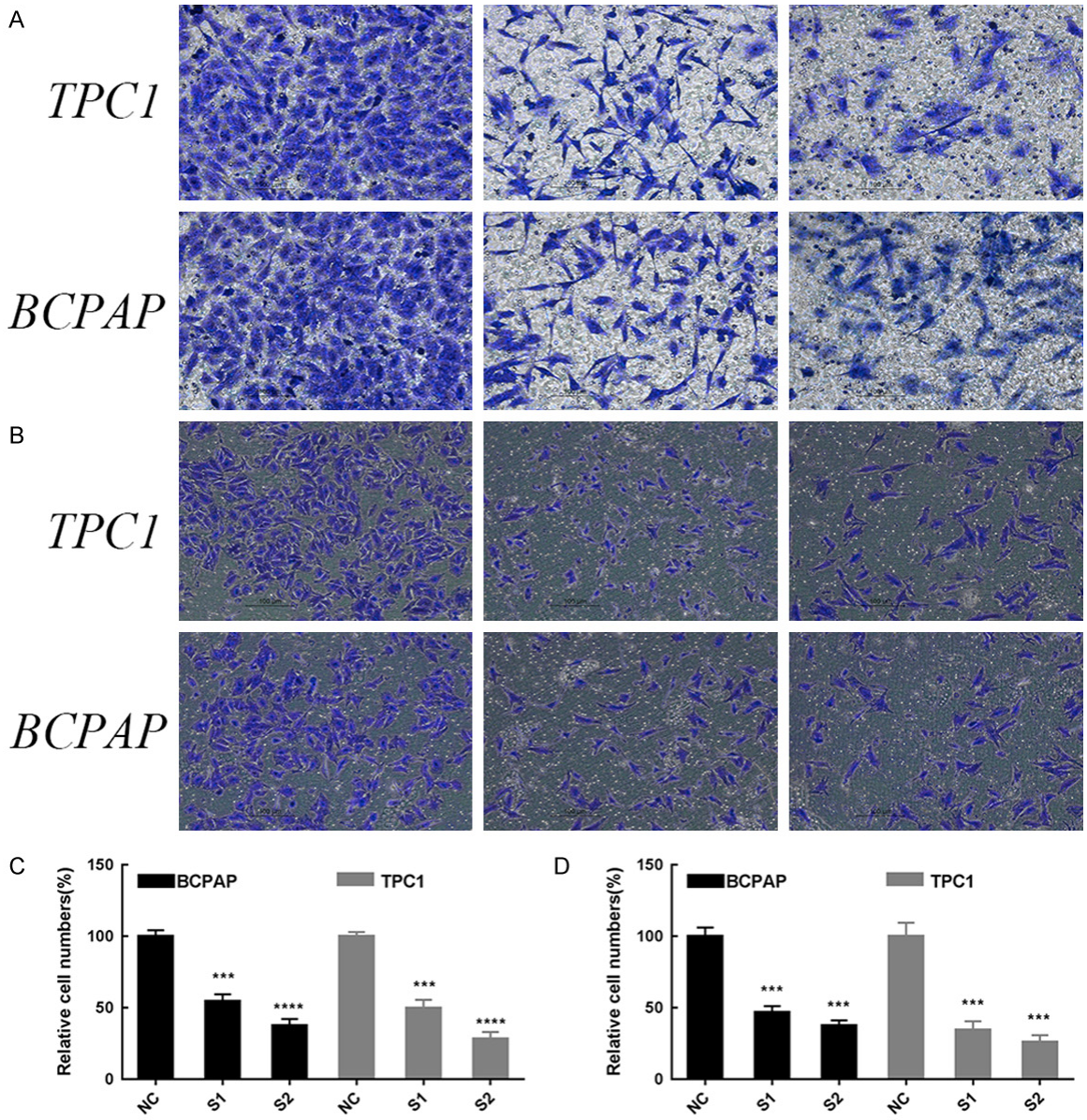

Figure 4. The downregulated of RTL4 inhibits migration and invasion in PTC. (A) Migration assay (magnification, ×10) and (C) relative quantification of migrating cell number. Compared with NC, the migrating cell number was significantly less in TPC1 and BCPAP cells with downregulated RTL4 expression. (B) Invasion assay (magnification, ×10) and (D) relative quantification of invading cell numbers. Compared with NC, the invading cell number was significantly less in TPC1 and BCPAP cells with downregulated RTL4 expression. ***P<0.001, ****P<0.0001.

suggest that RTL4 plays an important role in the TC and it might become a potential diagnostic and therapeutic molecular marker for PTC.

Conclusion

All in all, we found that RTL4 plays an important role in PTC. Logistic regression analysis showed that RTL4 was a risk factor for lymph node metastasis of PTC. In vitro experiment, the down-regulated RTL4 decreased cell proliferation and colony formation and inhibited migration and invasion. The experimental results were consistent with the clinical features. RTL4 may participate in the HOTAIR-miR-206-ZCCHC16 ceRNA regulatory network and be regulated and play a role in the ceRNA regulatory network. RTL4 may provide a potential therapeutic target for the treatment of PTC, as well as a biomarker for the assessment of PTC lymph node metastasis and the decision of the

surgical approach. It helps to achieve precision medicine of PTC.

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We submitted an ethical review to the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University and obtained permission. Written informed consent was issued by the patients for the publication of this research and accompanying images. A copy of the written consent is ready for review by the Editor in Chief of this journal.

Disclosure of conflict of interest

None.

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