Circular RNA PVT1 oncogene (CircPVT1) promotes the progression of papillary thyroid carcinoma by activating the Wnt/β-catenin signaling pathway and modulating the ratio of microRNA-195 (miR-195) to vascular endothelial growth factor A (VEGFA) expression

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ABSTRACT
Circular RNAs (circRNAs) have been reported to be involved in the progression of papillary thyroid carcinoma (PTC). However, the role of circular RNA PVT1 oncogene (circPVT1) in PTC has rarely been reported. In this study, we aimed to investigate the function and mechanism of circPVT1 in PTC. The expression level of circPVT1, miR-195 and VEGFA were determined by reverse transcription-quantitative PCR (RT-qPCR). Fisher's exact test was used to analyze the correlation between circPVT1 expression and PTC clinical features. Cell Counting Kit-8 (CCK-8) and 5-Ethynyl-2'-deoxyuridine (EdU) staining assay and transwell assay were conducted to evaluate the cell proliferation, migration and invasion ability. Dual-luciferase reporter and Western blot assay were conducted for evaluating the correlation between miR-195 and circPVT1 or VEGFA. The results of RT-PCR showed that the expression level of circPVT1 was significantly upregulated in PTC tissues and cell lines. After downregulating circPVT1 expression in PTC cells, the abilities of cell proliferation, migration, and invasion were obviously suppressed, and the Wnt/β-catenin signaling pathway was also repressed. Besides, miR-195 could both bind to PVT1 and VEGFA, while PVT1 could promote the expression of VEGFA by binding to miR-195. Downregulation of VEGFA expression in PTC cells revealed weakened cell proliferation, migration, and invasion capacities, and restrained Wnt/β-catenin signaling pathway. Therefore, we demonstrated that circPVT1 could promote VEGFA expression by sponging miR-195. CircPVT1 could serve as a molecule sponge for miR-195 and mediate the ceRNA network to promote the expression of VEGFA, thus contributed to the malignant progression of PTC.

Introduction
Thyroid cancer (TC) is one of most prevalent endocrine malignancy worldwide, and its incidence has doubled globally in the last 30 years [1–3]. TC is one of the reasons for the high incidence of malignant tumors in women, as it is the fifth most diagnosed malignant tumor in this group [4,5]. Papillary thyroid cancer (PTC) is the most common histotype of TC, accounting for approximately 85% of all TC [6]. Although the prognosis of PTC is generally good, a high quality of life still cannot be guaranteed. This is because some PTC patients show early epidural infiltration, lymphatic metastasis, and even distant metastasis or other high-risk complications [7]. More than 30% of PTC patients develop into lymph node metastasis [8], while 2.6% to 3.7% of patients suffer from distant metastasis [5,9]. The occurrence of distant metastasis and invasion of PTC indicates a poor prognosis, and often the disease cannot be effectively controlled [10]. Consequently, investigating the mechanism of the malignant progression of PTC has vital theoretical significance and potential clinical value.

Circular RNAs (circRNAs) were discovered approximately 40 years ago [11], but have not attracted widespread attention until recently. As circRNAs are ncRNAs, they were initially...
thought to be byproducts of pre-mRNA splicing errors [12]. Compared with other types of non-coding RNAs (ncRNAs), circRNAs have neither 5' to 3' polarity nor a polyA tail, because they form covalently closed loops. These circRNAs are more conserved and stable than linear ncRNAs [13,14]. In addition, some studies have found that there are four basic types of circRNAs [15]: exon circRNAs, intronic circRNAs, exon-intron circRNAs, and intergenic circRNAs. With the rapid development of next-generation sequencing technology, many circRNAs have been identified in mammalian transcriptomes and various cancers [16–18]. Because circRNA is not easily degraded by exonuclease RNase R, most of them have a long half-life and are widely present in tissues and peripheral blood, and they are highly conserved and have spatial tissue and disease specificity [19,20]. These characteristics endow it with a potential function, such as acting as a miRNA molecular sponge, regulating gene expression and participating in protein translation, and may also be a potential target for disease intervention [21,22]. In addition, circRNA in peripheral blood has stable biological properties and is expected to become a molecular marker for disease diagnosis and prognosis [16,23]. In human cancers, substantial researches have demonstrated that circRNAs may play an essential role in cancer growth, metastasis, and malignant progression. For example, Li et al. found that circRNA hsa_circ_0014130 can act as a miR-132-3p sponge for playing oncogenic roles in bladder cancer by upregulating potassium inwardly rectifying channel subfamily J member 12 (KCNJ12) expression [24]. Zhang et al. demonstrate that hsa_circ_0058106 can induce epithelial-mesenchymal transition (EMT) and metastasis in laryngeal cancer via sponging miR-153 and inducing twist family bHLH transcription factor 1 (TWIST1) nuclear translocation [25]. Cai et al. show that circ-NOL10 regulated by metadherin (MTDH)/CASC3 exon junction complex subunit (CASC3) can suppress breast cancer progression and metastasis via bind to multiple miRNAs and programmed cell death 4 (PDCD4) [26]. These studies identify that aberrant circRNAs expression may be encouraging diagnosis biomarkers and therapeutic target for PTC.

The circRNA PVT1 (circPVT1) is a recently identified circRNA situated in the known cancer susceptibility region chr8: 128902834–128903244 [27,28]. It originates from the third exon of the PVT1 and has been identified as an oncogene [27]. Previous studies have shown that circPVT1 may play a vital role in the initiation and progression of several tumors, including laryngeal cancer [29], non-small cell lung cancer [30], gastric cancer [31], clear cell renal cell carcinoma [32], ovarian cancer [33], cervical cancer [34] and breast cancer [35]. However, the expression pattern and biological function of circPVT1 in PTC remain unknown. In the current study, we hypothesized that circPVT1 might assume an essential role in the progression of PTC. The aim of this study was to reveal the biological function and potential molecular mechanism of circPVT1 regulating the progression of PTC. We firstly measured circPVT1 expression level in the PTC tissues and cell lines. Meanwhile, we detected the functions of circPVT1 on the cell growth, migration, and invasiveness of PTC. Finally, we revealed the underlying mechanism that circVRK1 sponged miR-195 and accelerated the VEGFA expression, so as to regulate the Wnt/β-catenin signaling pathway.

Materials and methods sample Collection

Fifty pairs of PTC tumor samples and matching contralateral normal samples were collected from patients with PTC in our hospital between October 2015 and March 2017. The samples were frozen in liquid nitrogen and stored at −80°C until use. We verified the cancer status of all the tissue samples by pathological examination. The Ethics Committee of the Tinglin Hospital of Jinshan District approved the study (2015–023) and informed consent was obtained from patients before surgical operation.

Cell culture

We purchased PTC cell lines (K1, IHH-4, BCPAP, and TPC-1) and human thyroid follicular epithelial cells (Nthy-ori 3–1) from the Hematology Institute (Beijing, China) of the Chinese
Academy of Chinese Medical Sciences. The K1, IHH-4 and Nthy-ori 3–1 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, Grand Island, NY, USA), while TPC-1 and BCPAP cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM). Additional MEM Non-Essential Amino Acids Solution (NEAA 100X) (Invitrogen, 11140050) was added to the BCPAP cell line. All medium was containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), penicillin (100 U/mL)/ streptomycin (100 µg/mL). The cells were incubated in a humidified incubator at 37°C and 5% CO2.

Cell transfection

We purchased the PVT1 small interfering RNA (siRNA), VEGFA siRNA, miR-195 inhibitor, and their corresponding negative controls from GenePharma (Shanghai, China). Next, we mixed the cells in a 6-well plate (5 × 10^4 cells per well). Following the manufacturer’s instructions, after the cell density reached 80%, the transfection reagent was mixed with Lipofectamine 2000 (Invitrogen) and added to the cells [36]. The mixture was incubated at room temperature for 30 min and subsequently transferred to a Petri dish. After transfection for 48 h using Lipofectamine 2000, the transfected cells were collected to assess the transfection efficiency by qRT-PCR. The sequences of primers for transfection were listed below: circPVT1 siRNA: 5’-GCAAAUAGAAAG CUACCAAUTT-3’; scramble siRNA: 5’-UUCUCCG AACGUGUCACGT-3’; VEGFA siRNA: 5’-CUG GAAUUGAUUAUUCUG-3’; scramble siRNA: 5’-UUCUCGAAACUGUACGT-3’; miR-195 mimics sense, 5’-UAGCAGCAAGAUAUUGG C-3’; miR-195 mimics antisense, 5’-CAUAUUUC UUGCUGCUAUU-3’; mimics negative control (miR-NC) sense, 5’-UUCUCGAAACUGUACGT TT-3’; miR-NC antisense, 5’-ACUGACACGUUCG GAGAATT-3’.

RNA transcription and reverse transcription-quantitative PCR (RT-qPCR)

After transfection for 24–48 h, purified total RNA was extracted from tissues and cells using TRIzol (Invitrogen). cDNA was obtained by reverse transcription in a 20 µL reaction system using the PrimeScript RT reagent Kit (TAKARA, Code No. RR037A) according to the manufacturer’s instructions. Real-time quantitative reverse transcription polymerase-chain reaction (RT-PCR) analysis was performed to determine transfection efficiency using the following conditions: 92°C for 10 min, followed by 40 cycles of 92°C for 10 s and then 60°C for 1 min [37]. GAPDH and U6 were used as the internal reference, respectively. The primer sequences used were as follows: PVT1 (F: 5’-GGTTCCACAGCAGTTATTC-3’; R: 5’-CAACTTCCTTGGGTCTCC-3’); miR-195 (F: 5’-ACGATGCCCAACGACAGCC-3’; R: 5’-AGCA CCATCGTCCGGCAGGCA-3’); VEGFA (F: 5’-AAT TTCTGCTGTTCGTGG-3’; R: 5’-TCTCGATTTGG ATGGCAGT-3’); Wnt3a (F: 5’-TCTACGACGGTC ACACCTG-3’; R: 5’-CCCTGCTTCCTCAGTGGAG T-3’); β-catenin (F: 5’-CAGCAGCAATTGTGGAG GG-3’; R: 5’-CAGCAGCAATTGTGGAG GG-3’); GAPDH (F: 5’-GGAATCCACTGGCGTCTTCA-3’; R: 5’-GGTTACGCCCCATCACAAAC-3’); U6 (F: 5’-CTCGTCCGCGCAACGCAC-3’; R: 5’-AAGCCTCTCA CGAATTTCG-3’).

Cell proliferation activity assay

We used Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) to detect cell growth ability and the method referred to the previous study [38]. Cells in the medium were digested with 0.25% trypsin and the cell suspensions were subsequently harvested and seeded into 96-well plates, with 6 wells per group. Each well contained at least 2 × 10^3 cells and 200 µL of the medium. The cells were incubated overnight for adherent growth and the cell supernatant was washed with phosphate-buffered saline. A mixture containing 90 µL of pure 1640 medium and 10 µL of CCK-8 solution (Beyotime Biotechnology, Shanghai, China) was added to each well. After 2 h of incubation, the absorbance of each well was measured at 450 nm using a microplate reader.

Transwell experiment

The cell invasiveness was examined with the 8-mm transwell (Millipore, Billerica, MA, USA) with (invasion assays) or without (migration assays) the coating of Matrigel. According to
previous study [39], the cells were seeded into serum-free medium and added to the upper chamber (1 × 10^4 cells per chamber) coated with 200 mg/mL of Matrigel. A medium containing 10% FBS was added to the lower chamber as a chemical attractant. After 24 h of incubation, the cells in the upper chamber were removed by wiping with a cotton swab. We then fixed the cells invading the lower surface of the filter in 70% ethanol for 30 min, and stained them for an additional 10 min with 0.2% crystal violet. The invading cells were photographed and counted across five random fields of view in each cell under an optical microscope at 200× magnification.

**5-ethyl-2'-deoxyuridine (EdU) assay**

The EdU assay was conducted according to previous study [40]. The cells were pre-inoculated in a 24-well plate at a density of 5 × 10^3 cells per well. The plated cells were then incubated in 4% methanol for 30 min, followed by permeabilization in 0.5% TritonX-100 for 10 min, and a 30-min reaction in 400 μL of 1x ApollorR. Afterward, the cells were stained with 4',6-diamidino-2-phenylindole for another 30 min. The EdU-stained cells were counted under a fluorescence microscope (CKX41-F32FL, Olympus, Tokyo, Japan) to calculate the percentage of EdU-positive cells.

**Western blot analysis**

The experimental method of Western blot referred to the previous literature [41]. Total protein was extracted from the transfected cells and the equal quantity proteins (30 μg) were separated using a 10% polyacrylamide gel and transferred to a 0.22 μm polyvinylidene fluoride membrane. The membranes were then incubated in blocking buffer (5% skim milk) for 2 h, and then incubated with primary antibodies at 4°C overnight. After incubation with the corresponding secondary antibodies for 2 h at room temperature, the protein bars were visualized by chemiluminescence using the ECL reagent (Pierce, Rockford, IL, USA). The antibodies used were as follows: Wnt3a (ab219412, 1/1000, abcam), β-catenin (ab32572, 1/5000, abcam), β-actin (ab8226, 1/1000, abcam), VEGFA (ab52917, 1/10000, abcam) and the anti-rabbit and antimouse peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology).

**Dual luciferase reporter experiment**

We used bioinformatics websites (starbase, https://starbase.sysu.edu.cn/) to predict the potential targets of miR-195, PVT1, and VEGFA [42]. A PVT1 3'-UTR or a mutant PVT1 3'-UTR with a predicted target site was inserted into a pGL3-promoter vector. Cells in the logarithmic growth phase were seeded into a 96-well plate at a density of 1.5 × 10^3 cells per well and cultured in an incubator for 24 h. After co-transfecting the cells for 48 h with a final concentration of 50 nmol/L of miRNA mimics or a non-target control, and 100 ng of the dual-reporter gene vector with a target gene 3' UTR or a mutant vector 3' UTR, the medium was aspirated and 35 μL fresh medium added per well. Next, 35 μL of luciferase (Promega, Madison, WI, USA) substrate was added to each well. After shaking for 10 min, the fluorescence intensity was measured. The experiment was repeated 3 times.

**Statistical analysis**

Data analysis was performed with the SPSS software (SPSS ver. 17.0, SPSS Inc., Chicago, IL, USA), and all the figures were processed with the GraphPad Prism software (Version X; La Jolla, CA, USA). Comparisons between two groups were analyzed using Student’s t-test. One-way analysis of variance (ANOVA) followed by Tukey’s test was utilized to analyze the comparisons among more than two groups. Fisher’s exact test was used to analyze the correlation between circPVT1 expression and PTC clinical features. The Kaplan–Meier method was applied to calculate the survival rate of patients. The results were displayed as mean ± standard deviation (SD). Statistical significance was set at p < 0.05.

**Results**

This study aimed to reveal the expression pattern, biological function and potential molecular mechanism of circPVT1 in PTC. We hypothesized that circPVT1 might act as a significant role in the
progression of PTC. In basis of our results of in vitro experiments, we demonstrated that circPVT1 was obviously up-regulated in osteosarcoma tissues and cell lines, and circPVT1 could sponge miR-195 to up-regulate VEGFA expression to promote PTC progression.

**CircPVT1 was highly expressed in cells from PTC tissue**

Through RT-PCR, we found that the level of PVT1 expression in PTC tissue was significantly higher than that in non-cancerous tissue Figure 1(a). Meanwhile, according to the median of circPVT1 level, we divided 50 cases into two groups, namely, Circ PVT1 High group (n = 25) and circPVT1 Low group (n = 25). The Kaplan–Meier curve indicated that the expression level of circPVT1 had no significant correlation with the overall survival rate of PTC patients (cut-off = 0.225, p > 0.05) Figure 1(b). We also analyzed the correlation between the expression levels of PVT1 and special clinical pathological parameters. As shown in Table 1, the expression level of PVT1 was significantly related to tumor size, TNM stage, and lymph node metastasis in patients with PTC. Compared to Nthy-ori 3–1 cells, the PVT1 expression level in PTC cell lines (K1, IHH-4, BCPAP, and TCP1) increased significantly Figure 1(c). We selected TPC-1 and BCPAP cells for further experiments. First of all, we transfected si-PVT1 into BCPAP cells and assessed the transfection efficiency. We observed a significant decrease in the PVT1 expression levels in the BCPAP cells due to this transfection Figure 1(d).

**Downregulation of PVT1 could inhibit the proliferation, migration, and invasion of PTC cells**

The effect of the PVT1 expression level on the PTC cells was detected using a CCK-8 test. After the
Table 1. Relationship between circPVT1 expression and the clinical pathological characteristics of PTC patients (n = 50).

| Clinic pathological features | NO. of cases | CircPVT1 (n, %) | p-value |
|------------------------------|--------------|-----------------|---------|
| Gender                       |              |                |         |
| Male                         | 29           | 14/15 11        | >0.05   |
| Female                       | 21           | 11/10           |         |
| Age                          |              |                |         |
| ≤45                          | 34           | 15/19           | >0.05   |
| >45                          | 16           | 7/9             |         |
| Extra thyroidal extension    |              |                |         |
| Negative                     | 17           | 7/10            | >0.05   |
| Tumor size                   |              |                |         |
| ≤1                           | 36           | 25/11           | <0.05   |
| >1                           | 14           | 4/10            |         |
| TNM stage                    |              |                |         |
| I/II                         | 28           | 20/8            | <0.05   |
| III/IV                       | 22           | 7/15            |         |
| Lymph node metastasis        |              |                |         |
| ≤45                          | 23           | 16/7            | <0.05   |
| >45                          | 27           | 9/18            |         |
| Nodular Goiter               |              |                |         |
| Negative                     | 31           | 15/16           | >0.05   |
| Positive                     | 19           | 9/10            |         |

**Reduction of PVT1 expression in TPC-1 and BCPAP cells**

The cell proliferation capacity was significantly inhibited Figure 2(a,b). Similar results were observed in the EdU experiment Figure 2(c,d). The transwell assay showed that after the downregulation of PVT1 expression, cell migration Figure 2(e,f), and invasiveness were also significantly reduced Figure 2(g,h). Based on the above observations, we concluded that reducing PVT1 expression can significantly inhibit the proliferation, migration, and invasion of PTC cells.

**Downregulation of PVT1 could inhibit the Wnt/β-catenin signaling pathway**

To further explore the specific role of PVT1 in PTC development, we lowered the expression of PVT1 in the PTC cells and detected the expression of genes related to the Wnt/β-catenin signaling pathway using RT-PCR. The results showed that following the inhibition of PVT1 expression, the mRNA expression of these genes was significantly decreased Figure 3(a,b). Furthermore, the level of proteins related to Wnt/β-catenin signaling pathway-related genes was also significantly reduced Figure 3(c). Thus, we found that following the inhibition of PVT1 expression, the expression of the Wnt/β-catenin signaling pathway was also significantly inhibited. This indicated that PVT1 could play a role in activating the Wnt/β-catenin signaling pathway.

**PVT1 could target miR-195 and regulate its expression**

We used bioinformatics to predict potential binding targets for circPVT1 and miR-195 Figure 4(a), and then constructed wild-type and mutant sequences of PVT1 (PVT1-WT and PVT1-MUT, respectively). Using dual-luciferase reporter gene experiments, we discovered that after the transfection of miR-195 mimics into the TPC-1 and BCPAP cells, the luciferase level in the PVT1-WT 3’UTR group was reduced, while there was no significant difference in the luciferase level in the PVT1-MUT 3’UTR group Figure 4(b,c). This result implied that PVT1 could target miR-195. To further study the regulatory relationship between the two, we transfected the cells with an miR-195 inhibitor. Using RT-PCR, we found that inhibition resulted in significantly decreased miR-195 expression levels after transfection Figure 4(d,e), and significantly increased PVT1 expression levels Figure 4(f). In addition, we found that miR-195 expression was significantly increased after PVT1 expression was downregulated Figure 4(d). Thus, we found that PVT1 could target miR-195 and inhibit its expression.

**PVT1 could regulate VEGFA expression through miR-195**

We also used bioinformatics to predict potential binding targets for miR-195 and VEGFA Figure 5(a) and then constructed VEGFA wild-type and mutant sequences (VEGFA-WT and VEGFA-MUT, respectively). Using a dual luciferase reporter gene experiment we found that, after transfection of miR-195 mimics into TPC-1 and BCPAP cells, the luciferase levels in the VEGFA-WT 3’UTR group decreased, while those in the VEGFA-MUT 3’UTR showed no significant difference Figure 5(b,c). This suggested that miR-195 can target VEGFA. To further study the regulatory relationship between the two, we transfected cells with si-VEGFA to inhibit its expression. RT-PCR showed that following transfection, the VEGFA expression level in the cells was significantly reduced Figure 5(d), while the miR-195 expression level significantly increased Figure 5(e), and the
PVT1 expression level decreased significantly Figure 5 (f). To further explore this regulatory behavior, we used RT-PCR to show that VEGFA expression levels increased after downregulation of miR-195 expression Figure 5(g), and also that VEGFA expression was significantly suppressed after PVT1 expression was suppressed Figure 5(h). Western blot analysis also showed the same results at the protein level Figure 5 (i). Based on the above results, we concluded that PVT1 could regulate the expression of VEGFA.
through targeted binding with miR-195 in the PTC cells.

**Lowering VEGFA restrained the growth, migration, and invasion of PTC cells**

In order to further explore the specific role of VEGFA in the development of PTC, we tested its influence on cell proliferation capacity via a CCK-8 test. The results suggested that the cell proliferation capacity was significantly inhibited Figure 6(a, b) after the reduction of VEGFA expression in the TPC-1 and BCPAP cells. The EdU experiment revealed the same outcome Figure 6(c,d). We used the transwell assay to detect cell migration and invasion and found that, after reducing VEGFA expression, the cell migration capacity was significantly reduced Figure 6(e,f) and the invasion capacity was also significantly inhibited Figure 6(g,h). Thus, we concluded that reduced VEGFA expression can significantly inhibit the proliferation, migration, and invasion of PTC cells.

**Downregulation of VEGFA inhibited the Wnt/β-catenin signaling pathway**

In order to further elucidate the mechanism by which VEGFA is involved in the development of PTC, we tested the expression of Wnt/β-catenin signaling pathway-related genes through RT-PCR after lowering VEGFA expression in the cells. Upon lowering the VEGFA expression, the level of mRNA expression for the Wnt/β-catenin signaling pathway-related genes decreased significantly Figure 7(a,b), and the Western blot also showed significantly lower Wnt/β-catenin signaling pathway-related protein levels Figure 7(c). Taken together, we found that the Wnt/β-catenin signaling pathway-related gene expression also significantly inhibited VEGFA expression, and that PVT1 activated the Wnt/β-catenin signaling pathway by targeting miR-195 to upregulate VEGFA expression.

**Discussion**

In recent decades, the incidence of TC has been increasing, and it is the most common incretory tumor [43,44]. PTC is the main type of TC, accounting for approximately 90% of cases and with an overall 5-year survival rate of approximately 97% [45,46]. However, the clinical prognosis of late PTC is poor, partly because the molecular mechanism of PTC occurrence and development is not adequately understood. Therefore, finding new biomarkers and therapeutic targets to improve the diagnosis and therapeutic outcomes of PTC is a clinical problem.

Many ncRNAs have been shown to regulate the expression of genes in cancer cells and are expected to become molecular biomarkers of cancer [12,47]. The structure of circRNAs has been shown to be highly stable and resistant to RNA degradation pathways. This suggests that circRNAs may be more technically suitable as molecular biomarkers for human cancer. In recent years, many studies have shown that circRNAs are involved in the initiation and development of PTC. Luo et al. identify that has_circ_0001018 can promote PTC by facilitating cell survival invasion, G/S cell cycle progression, and repress cell apoptosis via crosstalk with miR-338-3p and SRY-box transcription factor 4 (SOX4) [48]. Chu et al
show that circular RNA circRUNX1 can promote PTC progression and metastasis by sponging miR-296-3p and regulate DHD domain containing 2 (DDHD2) expression [49]. Ye and colleagues have demonstrated that circular RNA circFOXM1 may play a vital role in PTC by sponging miR-1179.
Figure 5 miR-195 could target VEGFA. a. Bioinformatics predicts the potential binding sites between miR-195 and VEGFA; b. Dual luciferase reporting gene experiment in TPC-1 cells shows that miR-195 can target VEGFA; c. Dual luciferase reporting gene experiment in BCPAP cells shows that miR-195 can target VEGFA; d. After transfecting si-VEGFA into TPC-1 as well as BCPAP cells, VEGFA expression was reduced; e. MiR-195 expression levels rises after down-regulating expression of VEGFA in TPC-1 and BCPAP cells; f. PVT1 expression level declines after VEGFA expression is lowered in TPC-1 and BCPAP cells; g. VEGFA expression levels rose after downward expression in TPC-1 and BCPAP cells; h. The expression of VEGFA is reduced after PVT1 expression is reduced in TPC-1 and BCPAP cells; i. Western blot shows that when lowering PVT1 expression in TPC-1 as well as BCPAP cells, VEGFA protein levels decrease; when miR-195 is downgraded, VEGFA protein levels increase. (** P < 0.01, the expression level of VEGFA was normalized to GAPDH).
and regulate high mobility group box 1 (HMGB1) expression [50].

In this study, we found that circPVT1 had significantly increased expression in PTC tissue. Specifically, PVT1 expression level had a significant correlation with tumor size, TNM stage, and lymphatic metastasis in patients with PTC. In addition, we also found that circPVT1 expression level was significantly increased in the PTC cell lines compared to the Nthy-ori 3–1 cells. However, due to the limited number of specimens we collected and the short
follow-up time, we have not yet observed the correlation between the expression level of CircPVT1 and the prognosis of PTC patients. At present, we have collected more specimens, and follow-up information will be improved. We will further verify the potential of circPVT1 as a diagnostic marker for PTC.

Then, we detected the effect of circPVT1 on cell proliferation, migration, and invasion. After reducing the expression level of circPVT1 in PTC cells, the ability of cell proliferation, migration, and invasion were significantly inhibited, and the Wnt/β-catenin signaling pathway was also inhibited. The Wnt/β-catenin signaling pathway, also known as the classic Wnt signaling pathway, has been studied in-depth [51,52]. Activation of the classic Wnt signaling pathway can contribute to extracellular Wnt combining with the transmembrane receptors LRPS/6 and Fzd to form a complex. These receptors then become activated, which in turn activates the intracellular Dishevel protein, causing the inhibition of glycogen synthase enzyme-3 activity and subsequent detachment from the axin protein. This prevents formation of the β-catenin degradation complex, which in turn, prevents phosphorylation and degradation of β-catenin, thereby increasing the intracellular β-catenin expression. When β-catenin reaches a certain level, free β-catenin undergoes nuclear transfer and binds to the transcription factor TCF/LEF to form a transcription-activated complex. This complex eventually enhances or weakens the expression of certain genes. The Wnt/β-catenin signaling pathway has also been proved to be involved in the progression of PTC [53–55].

Previous studies documented that circRNAs were involved in regulating diverse biological processes via functioning as competing endogenous RNAs for miRNAs [37,38]. CircPVT1 has been proved to locate mainly in cytoplasm, which meant that it might act as microRNA sponges to regulate downstream gene expression. MiR-195 is dysregulated in several tumors, such as colon cancer [56], non-small lung cancer [57], laryngeal cancer [58,59], gastric cancer [60], cervical cancer [61], colorectal cancer [62], breast cancer [63], lung adenocarcinoma [64] and osteosarcoma [65]. Moreover, miR-195 has been demonstrated that play a significant role in PTC [66–69]. VEGFA is a powerful and specific endothelial cell fissure agent that, in combination with receptors, can participate in promoting the division and proliferation of vascular endothelial cells and increase microvascular permeability [70,71]. VEGFA has been shown to play an important role in tumor development. VEGFA is not only important to endothelial cell survival but also helps circulating endothelial cells reach new blood vessels. This in turn can promote tumor growth and angiogenesis, and thus VEGFA expression may become a new tumor biomarker [72,73]. VEGFA also participates in the development of PTC [74]. In this work, by using the dual luciferase reporter gene and Western blotting, we found that circPVT1 expression can be increased by targeting miR-195 to increase VEGFA expression. We have also shown in numerous experiments that the Wnt/β-catenin signaling pathway was inhibited and the cell proliferation, migration, and invasion abilities decreased after the reduction of VEGFA.
expression in cells. In summary, we found that circPVT1 could activate the Wnt/β-catenin signaling pathway to promote the development of PTC.

Of course, this article still has many shortcomings. First of all, we have not conducted in vivo experiments to further verify the biological effects and molecular mechanisms of circPVT1. Secondly, there are multiple binding sites for miRNAs on circPVT1. It remains to be explored whether circPVT1 binds to which miRNA plays the most important role. Thirdly, many studies have reported that ncRNAs have the potential to be developed as reagents for clinical treatment [75,76], whether circPVT1 can be used as a therapeutic target for corresponding research and development still needs a lot of experimental proof. Moreover, we also need to collect more clinical specimens to explore the clinical application potential of circPVT1.

**Conclusion**

Overall, our study demonstrated that circPVT1 expression was frequently increased in PTC and high expression level of circPVT1 was related to tumor growth and metastasis and poor prognosis. From the perspective of mechanism, circPVT1 could function as a molecule sponge for miR-195 and mediated the ceRNA network to promote the expression of VEGFA, finally promoted the malignant progression of PTC. Therefore, the circPVT1/miR-195/VEGFA axis might be considered as a novel biomarker for prognosis and a promising therapeutic target for PTC treatment. Nonetheless, the current study only presents a theoretical basis for the mechanism of circPVT1 involvement in PTC. Further research is required to explore the specific mechanisms involving various other molecular pathways involved in PTC carcinogenesis.

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**Data Availability Statement**

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

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