Circular RNA PVT1 promotes progression of thyroid cancer by competitively binding miR-384

TEBO HUA and YONG LUO

Department of Thyroid Breast Surgery, Ningbo Medical Centre Lihuili Hospital, Ningbo, Zhejiang 315040, P.R. China

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Abstract. Plasmacytoma variant translocation 1 circular RNA (circPVT1) is involved in the initiation and development of several types of cancer. However, the underlying molecular role of circPVT1 in tumorigenesis of thyroid cancer remains to be elucidated. In the present study, relative expression of circPVT1 was markedly upregulated in thyroid cancer compared with adjacent normal tissue. circPVT1 expression was associated with clinical stage and lymph node metastasis. Furthermore, Cell Counting Kit-8, colony formation and Transwell chamber assays demonstrated that knockdown of circPVT1 decreased proliferation, migration and invasion of thyroid cancer cells *in vitro*. Moreover, circPVT1 directly interacted with microRNA (miR)-384, as shown by bioinformatics prediction and dual luciferase and RNA pull-down assay. miR-384 inhibition partially reversed the circPVT1 knockdown-mediated inhibitory effect on proliferation, migration and invasion of thyroid cancer cells. In summary, these findings demonstrated that circPVT1 may be a potential therapeutic target for thyroid cancer.

Introduction

Thyroid cancer has received considerable attention globally due to its increasing incidence rate (1). Most patients with thyroid cancer display a favorable prognosis following treatment with surgery, radiotherapy and chemotherapy (2). However, risk of recurrence is high because of metastasis (3). Therefore, it is important to understand the molecular mechanism of thyroid cancer progression to identify potential therapeutic targets.

Circular (circ)RNAs, a class of single-stranded closed loop endogenous non-coding RNA, do not encode protein (4). The function of circRNA is an important research topic in recent clinical studies since they are involved in the progression of multiple types of cancer (5,6). circRNAs are involved in the regulation of physiological and pathological cell processes by competitively binding to microRNAs (miRNAs or miRs), thereby reversing miR-mediated inhibition of downstream mRNA protein translation (7,8). A number of studies have shown that circRNAs serve an important role in tumorigenesis and function as oncogenes or tumor suppressors (9,10). A small number of circRNAs have been identified as diagnostic markers or therapeutics targets for thyroid cancer (11,12).

Plasmacytoma variant translocation 1 circular RNA (circPVT1), which is located on chromosome 8q24 (13), is considered to serve an oncogenic role in several types of cancer including liver (14), breast (15) and non-small cell lung cancer (16), osteosarcoma (17), glioblastoma (18), esophageal (19) and oral (20) and head and neck squamous cell carcinoma (21), as well as gastric cancer (22). However, the functional role and underlying mechanism of circRNA in thyroid cancer remain unclear. Thus, the present study aimed to investigate the expression and biological role of circPVT1 and elucidate its underlying regulatory mechanism in thyroid cancer.

Materials and methods

Patients and tissue samples. A total of 36 thyroid cancer and corresponding adjacent (distance, ~5 cm) normal tissue (ANT) samples were obtained from patients with thyroid cancer (mean age, 52.1 years; range, 24-72 years; 12 male; 24 female) at Ningbo Medical Centre Lihuili Hospital (Ningbo, China). All procedures were performed with the approval of the Ethics Committee of the Ningbo Medical Centre Lihuili Hospital (approval no. KY2022SL277-01) and written informed consent was obtained from all patients. All experiments were performed in accordance with the Declaration of Helsinki. None of the patients received any radiotherapy, chemotherapy or other therapy before undergoing surgery. All tissue samples were immediately snap frozen and stored at -80˚C until further use. Clinical information of patients is displayed in Table I.

Cell culture and transfection. TPC-1 cells were purchased from the Chinese Academy of Sciences and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37˚C with 5% CO_2.

Correspondence to: Dr Tebo Hua, Department of Thyroid Breast Surgery, Ningbo Medical Centre Lihuili Hospital, 57 Xingning Road, Yinzhou, Ningbo, Zhejiang 315040, P.R. China
E-mail: botehua313@tom.com

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miR-384 mimi cs (miR-384; 5'-AUUCCUAGAAAUUGUUCAUA-3'), inhibitors (anti-miR-384; 5'-UAUGAACAAUUUCUAGGAAU-3'), scrambled negative control (NC; 5'-UUCUCCGAACGUGUCACGU-3') oligos miR-NC and anti-miR-NC (5'-CAGUACUUUUGUGUAGUACAA-3'), pGPH1 plasmid containing short hairpin (sh)RNA targeting circPVT1 (sh-circPVT1; 5'-UGGGCUUGAGGCCUGAUCU-3') and pGPH1 plasmid containing scrambled control shRNA (sh-NC; 5'-UUCUCCGAACGUGUCACGUTT-3') were purchased from Shanghai GenePharma Co., Ltd. A total of $5x10^5$ TPC-1 cells was plated in 96-well plates and incubated for 24 h at 37˚C. The plasmids (1 μg/μl), mimics (100 nM) and inhibitors (100 nM) were transfected into TPC-1 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. After 48, the transfection efficiency was assessed.

Stable TPC-1 cells with sh-NC or sh-circPVT1 were selected using 400 ug/ml Geneticin (G418,Thermo Fisher Scientific, Inc.) and 200 μg/ml G418 as the maintenance concentration.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from thyroid cancer and ANT samples and cultured cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Nucleus and cytoplasm of TPC-1 cells were isolated using a PARIS extraction kit (cat no. AM1556; Thermo Fisher Scientific, Inc.). Complementary DNA was synthesized from 1 μg total RNA using PrimeScript RT (Takara Biotechnology Co., Ltd.) or All-in-one™ miRNA FirstStrand cDNA Synthesis kit (GeneCopoeia, Inc.). RT-qPCR was performed using SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.) and SYBR PrimeScript miRNA RT-PCR kit (Takara Biotechnology Co., Ltd.) for detecting circPVT1 and miR-384, respectively. Thermocycling conditions were as follows: Initial denaturation of 40 sec at 95˚C; followed by 40 cycles of denaturation at 95˚C for 5 sec and annealing and extension at 58˚C for 40 sec. GAPDH and U6 were chosen as the internal control for detection of circPVT1 and miR-384, respectively.

The primer sequences were as follows: circPVT1 forward, 5'-ATCGGTGCCTCAGCGTTCGG-3' and reverse, 5'-CTGTCCTCGCCGTCACACCG-3'; human GAPDH forward, 5'-ATCAATGGAAATCCCATCACCA-3' and reverse, 5'-GACTCCACGACGTACTCAGCG-3'; miR-384 forward, 5'-TGTTAAATCAGGAATTTTAA-3' and reverse, 5'-TGTTACAGGCATTATGAA-3' and U6 forward, 5'-CTCGTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The relative expression levels were calculated using $2^{-\Delta\Delta Cq}$ method (23).

Cell counting kit-8 (CCK-8) assay. CCK-8 assay was performed to assess cell proliferation. Briefly, transfected cells ($5x10^3$ cells/well) were seeded into 96-well plates at 37˚C for 24-72 h. A total of 10 μl CCK-8 regent (Dojindo Molecular Technologies, Inc.) was added into each well at indicated time and incubated for 2 h at 37˚C. Finally, optical density at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc.).

Plate clone formation assay. Exponentially growing cells (1,000 cells/well) were seeded into a 6-well plate and cultured in DMEM at 37˚C with 5% CO_2 for 10 days to form colonies. Subsequently, cells were stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA) for 15 min at 25˚C after being fixed with 4% paraformaldehyde for 30 min at 25˚C. Finally, the number of clones (>50 cells) was counted under a light microscope (X71; Olympus Corporation; magnification, x40).

Transwell assay. Transwell plates (Corning, Inc.) were employed to determine cell invasion and migration. The upper chambers were pre-coated with 50 μl 2.5 mg/ml Matrigel at 37˚C for 2 h (Corning, Inc.) for the invasion assays or uncoated for the migration assays. A total of $4x10^5$ transfected cells resuspended in serum-free DMEM were plated into the upper chamber DMEM supplemented with 20% FBS was added to the lower chamber. Following incubation for 24 h at 37˚C with 5% CO_2, the invaded and migrated cells were fixed with 4% paraformaldehyde at 25˚C for 30 min. and stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA) at 25˚C for 10 min. The stained cells were counted in five randomly selected fields of view using an inverted light microscope (Olympus Corporation).

Target prediction analysis. The potential target miRs of circPVT1 were predicted using The Encyclopedia of RNA Interactomes (ENCORI; starbase.sysu.edu.cn).

Dual-luciferase reporter assay. Wild-type (WT; 5'-UUUCUAGACUUGCUAGGAAA-3') or mutant type (MT, 5'-UUUCUUGACAAGGAUCCUUA-3') circPVT1 3' untranslated regions containing the predicted binding site for miR-384 were synthesized by Shanghai GenePharma Co., Ltd, and were cloned into the pmirGLO luciferase reporter vector (Promega Corporation) to generate circPVT1-WT or circPVT1-MT, respectively. The recombinant vectors circPVT1-WT or circPVT1-MUT and miR-384 mimics or miR-NC mimics were co-transfected into the TPC-1 cells using Lipofectamine® 2000 and cultured for 48 h. Dual-Luciferase Reporter Assay System (Promega Corporation) was used to detect luciferase activity. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

RNA pull-down assay. biotinylated WT miR-384 (WT-bio-miR-384), biotinylated MT miR-384 (MT-bio-miR-384) or control probe Bio-NC were purchased from Sangon Biotech Co. The biotinylated RNA was transfected into TPC-1 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and cultured at 37˚C with 5% CO_2 for 48 h according to the manufacturer's instructions. A total of ~$1x10^7$ cells were dissolved in the soft lysis buffer with 80 U/ml RNasin (Promega Corporation). The cell lysate were precipitated with M-280 streptavidin beads (Sigma-Aldrich; Merck KGaA) at 4˚C for 12 h. The beads were collected by centrifugation at 13,000 g for 10 min at 4˚C and cell debris was discarded. The bound RNAs were purified using RNeasy Mini kit (Qiagen GmbH). The relative expression of circPVT1 was measured via RT-qPCR.

Statistical analysis. At least three independent replicates were performed and data are presented as the mean ± SD. Data were

Table I. Association between clinicopathological features and circPVT1 expression in 36 patients with papillary thyroid cancer.

Variable	Cases	circPVT1 expression		P-value
		High	Low	
Age, years				0.3053
<45	14	5	9	
≥45	22	13	9	
Sex				0.7247
Male	12	5	7	
Female	24	13	11	
TNM stage				0.0408
I-II	28	11	17	
III-IV	8	7	1	
Tumor size, cm				0.1642
<1	23	9	14	
≥1	13	9	4	
Lymph node metastasis				0.0072
No	26	9	17	
Yes	10	9	1	

circPVT1, plasmacytoma variant translocation 1 circular RNA.

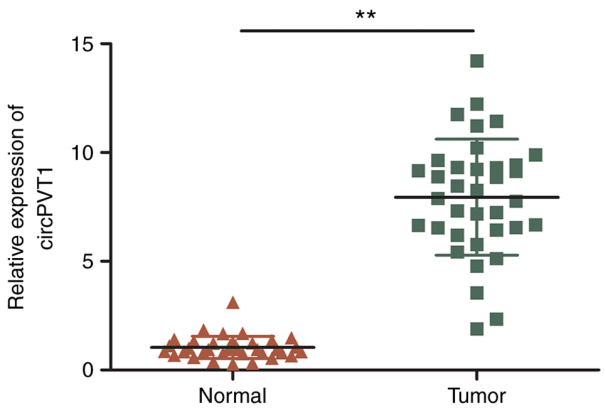

Figure 1. circPVT1 is upregulated in human thyroid cancer tissue. Relative expression of circPVT1 in 36 paired human thyroid cancer and adjacent normal tissue samples. Data are presented as the mean ± standard deviation. All experiments were conducted in triplicate. **P<0.01. circPVT1, plasmacytoma variant translocation 1 circular RNA.

analyzed using SPSS v 22.0 software (IBM Corp.). Differences between two or three groups were analyzed using parametric paired Student's t-test (two groups) or one-way analysis of variance followed by Bonferroni's post hoc test (three groups). The correlation between circPVT1 and miR-384 in thyroid cancer tissue was determined by Pearson's correlation coefficient analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

circPVT1 is upregulated in human thyroid cancer tissue. Relative expression of circPVT1 in 36 thyroid cancer and corresponding ANT samples was detected using RT-qPCR. The results revealed that the relative expression level of circPVT1 was significantly increased in thyroid cancer tissues compared with ANT (Fig. 1). Further analysis of the association between circPVT1 expression and clinicopathological features of patients with thyroid cancer demonstrated that high circPVT1 expression was positively associated with lymph node metastasis and advanced tumor-node-metastasis stage (TNM; Table I) but not with sex, age or tumor size (Table I).

circPVT1 knockdown inhibits proliferation and colony formation of thyroid cancer cells. As increased circPVT1 expression found in thyroid cancer tissue, the biological role of circPVT1 in thyroid cancer was investigated. TPC-1 cells were transfected with sh-NC vector or sh-circPVT1 and expression of circPVT1 was measured using RT-qPCR. Transfection with sh-circPVT1 decreased circPVT1 expression in TPC-1 cells compared with that in cells transfected with sh-NC, suggesting that circPVT1 was successfully knocked down in TPC-1 cells (Fig. 2A). CCK-8 assay demonstrated that circPVT1-knockdown significantly decreased cell proliferation in a time-dependent manner compared with sh-NC group (Fig. 2B). In addition, circPVT1 depletion decreased colony formation in TPC-1 cells compared with the sh-NC group (Fig. 2C).

circPVT1 knockdown inhibits migration and invasion of thyroid cancer cells. The biological effects of circPVT1 on thyroid cancer cell migration and invasion were evaluated. Transwell assay indicated that circPVT1-knockdown significantly reduced the number of migrated and invaded cells compared with sh-NC (Fig. 3A and B), suggesting that circPVT1-knockdown resulted in decreased cell migration and invasion.

miR-384 is a direct target of circPVT1 in THYROID CANCER cells. circPVT1 was primarily distributed in the cytoplasm of TPC-1 cells (Fig. 4A). It was hypothesized that circPVT1 interacted with miRs; miRs that bind to circPVT1 were selected using ENCORI. Among these miRs, miR-384, a tumor suppressive miR (24-26), was selected as a target of circPVT1 (Fig. 4B). To test this prediction, dual-luciferase reporter assay was performed; luciferase activity was decreased in TPC-1 cells co-transfected with circ-PVT1-WT and miR-384 mimics compared with that in TPC-1 cells co-transfected with circ-PVT1-WT and miR-NC (Fig. 4C) but remained unchanged following co-transfection with circ-PVT1-MT and miR-384 mimics or miR-NC (Fig. 4C). Compared with that wt-bio-miR-NC, RNA pull-down assay using wt-bio-miR-284 indicated that circPVT1 was significantly enriched in miR-384 mimics-precipitated RNA transcripts in TPC-1 cells (Fig. 4D). Moreover, RT-qPCR showed that relative expression of miR-384 was significantly upregulated in TPC-1 cells transfected with sh-circPVT1 compared with that in in TPC-1 cells transfected with sh-NC (Fig. 4E). Furthermore, miR-384 expression was downregulated in thyroid cancer tissue (Fig. 4F), and its

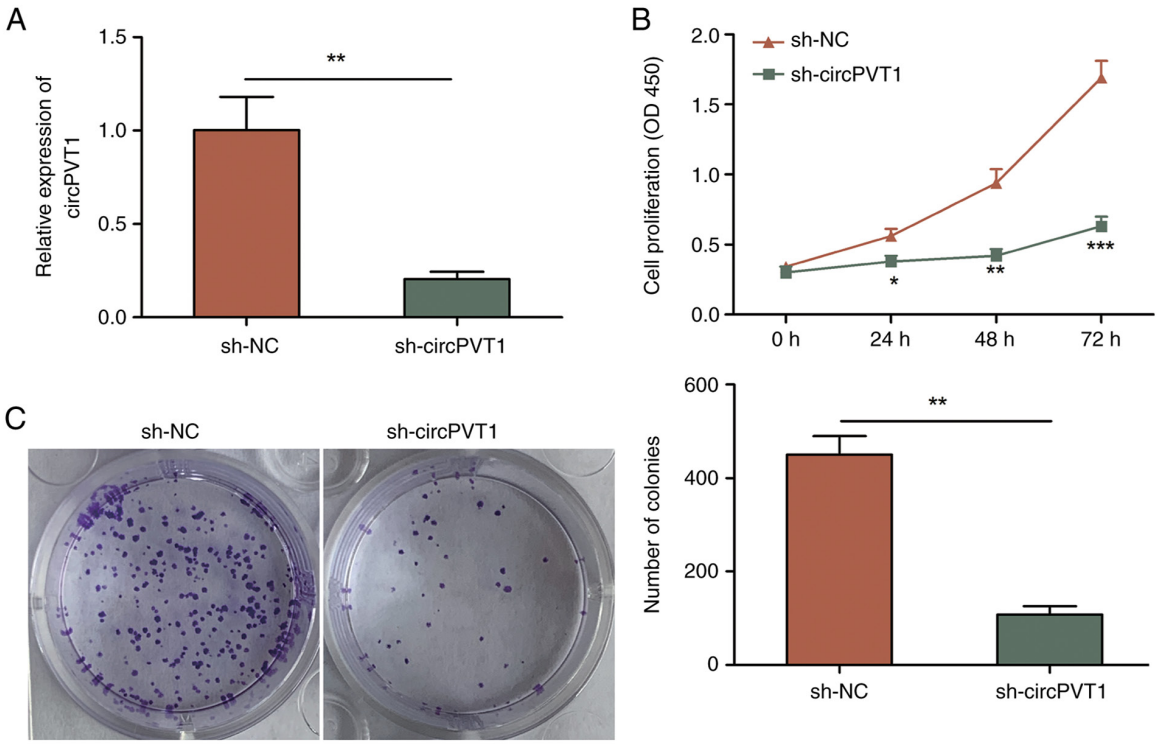

Figure 2. Knockdown of circPVT1 inhibits proliferation and colony formation of thyroid cancer cells. (A) Relative expression of circPVT1 in TPC-1 cells transfected with sh-NC or sh-circPVT1. (B) Proliferation and (C) colony formation of TPC-1 cells transfected with sh-NC or sh-circPVT1. Data are presented as the mean ± standard deviation. All experiments were conducted in triplicate. *P<0.05 and **P<0.01 vs. sh-NC. CircPVT1, plasmacytoma variant translocation 1 circular RNA; sh, short harpin; NC, negative control; OD, optical density.

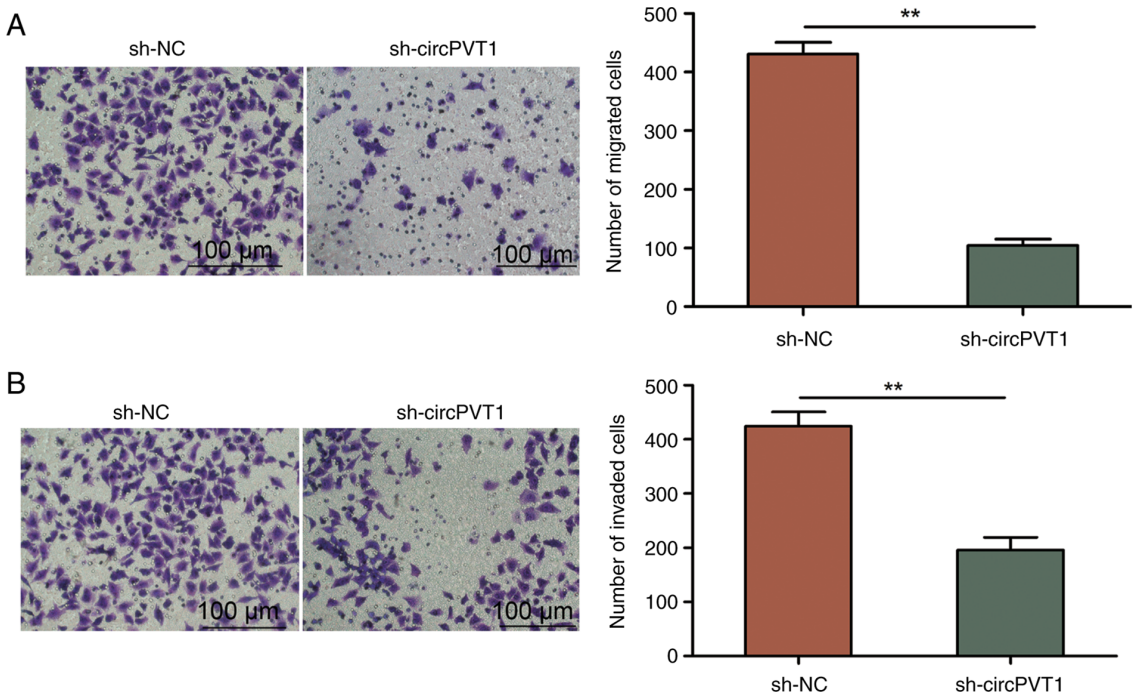

Figure 3. Knockdown of circPVT1 inhibits migration and invasion of thyroid cancer cells. (A) Migration and (B) invasion of TPC-1 cells transfected with sh-NC or sh-circPVT1 using Transwell/Matrigel assays. Data are presented as the mean ± standard deviation. All experiments were conducted in triplicate. **P<0.01 vs. sh-NC. circPVT1, plasmacytoma variant translocation 1 circular RNA; sh, short harpin; NC, negative control.

expression was negatively correlated with circPVT1 in thyroid cancer tissue (r=-0.5406 Fig. 4G). These results suggested that circPVT1 bound to miR-384 in thyroid cancer cells.

circPVT1 promotes thyroid cancer cell proliferation and invasion by sponging miR-384. Transfection of sh-circPVT1 significantly increased expression of miR-384 in TPC-1 cells

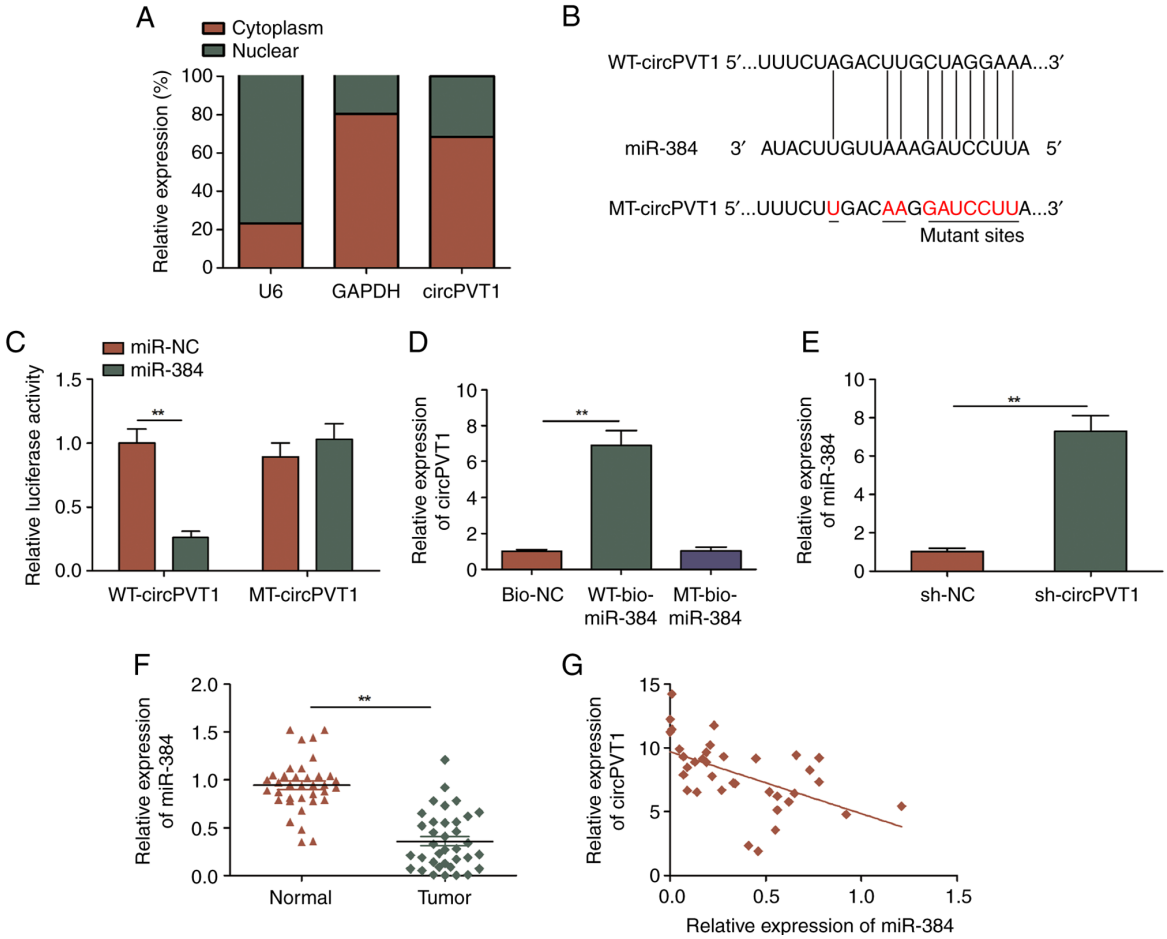

Figure 4. miR-384 is a direct target of circPVT1 in thyroid cancer cells. (A) Verification of circPVT1 subcellular localization. (B) Predicted miR-384 binding sites in WT-circPVT1 and designed MT-circPVT1 sequence. (C) Dual-luciferase reporter gene assays for binding of circPVT1 and miR-384 in TPC-1 cells. (D) Binding association between circPVT1 and miR-384 via RNA-pull down assay. (E) Relative expression of miR-384 in TPC-1 cells transfected with sh-NC or sh-circPVT1. (F) Relative expression of circPVT1 in 36 paired human thyroid cancer and adjacent normal tissue samples. (G) Spearman correlation analysis between circPVT1 and miR-384 expression in 36 thyroid cancer tissue samples. Data are presented as the mean ± standard deviation. All experiments were conducted in triplicate. **P<0.01 vs. sh-NC. circPVT1, plasmacytoma variant translocation 1 circular RNA; sh, short harpin; NC, negative control; miR, microRNA; WT, wild type; MT, mutant type; bio, biotinylated.

compared with that in TPC-1 cells transfected with sh-NC, whereas transfection of miR-384 inhibitor partially reversed this trend (Fig. 5A). Moreover, inhibition of miR-384 partly reversed circPVT1 depletion-mediated inhibitory effect on thyroid cancer cell proliferation, colony formation, migration and invasion (Fig. 5B-E). Collectively, the present findings suggest that miR-384 contributed to circPVT1-mediated thyroid cancercell proliferation and migration.

Discussion

A number of studies have demonstrated that circRNAs serve as diagnostic markers and therapeutic targets for thyroid cancer and are involved in biological processes (11,12). For example, Chen *et al* (27) found that circRNA_NEK6 promotes thyroid cancerprogression by targeting miR-370-3p to regulate Wnt signaling pathway. Yao *et al* (28) demonstrated that Hsa_circ_0058124 promotes proliferation and metastasis of thyroid cancer cells via the NOTCH3/GATA zinc finger domain containing 2A axis. Wang *et al* (29) showed that circRNA circ-ITCH serves as a tumor suppressor in thyroid cancer via regulating the miR-22-3p/CBL/β-catenin pathway. Fan *et al* (30) found that circ_0000144 promotes pathogenesis of thyroid cancer by sponging miR-217 to upregulate AKT3 expression. In the present study, circPVT1 was determined to function as an oncogenic circRNA in thyroid cancer by binding with miR-384.

circPVT1, derived from the PVT1 gene locus, was initially identified as a senescence-associated circRNA involved in regulating the proliferation and senescence of fibroblasts by sponging the let-7 miR family (13). circPVT1 is confirmed to have an oncogenic role in multiple types of cancer (14-22). For example, silencing of circPVT1 inhibits proliferation of hepatocellular carcinoma cells by downregulation of TRIM23 via miRNA-377 (14). circPVT1 promotes oral squamous cell carcinoma proliferation by sponging miR-125b (17). circPVT1 is involved in the progression of breast cancer by regulating the miR-29a-3p-mediated anterior gradient 2/hypoxia inducible factor-1α pathway (15). However, the function and underling mechanism of thyroid cancer remain unclear. In the present study, circPVT1 expression was higher in thyroid cancer than in matched normal tissue. Increased circPVT1 expression was associated with lymph node metastasis and advanced TNM stage. Moreover, functional experiments showed that

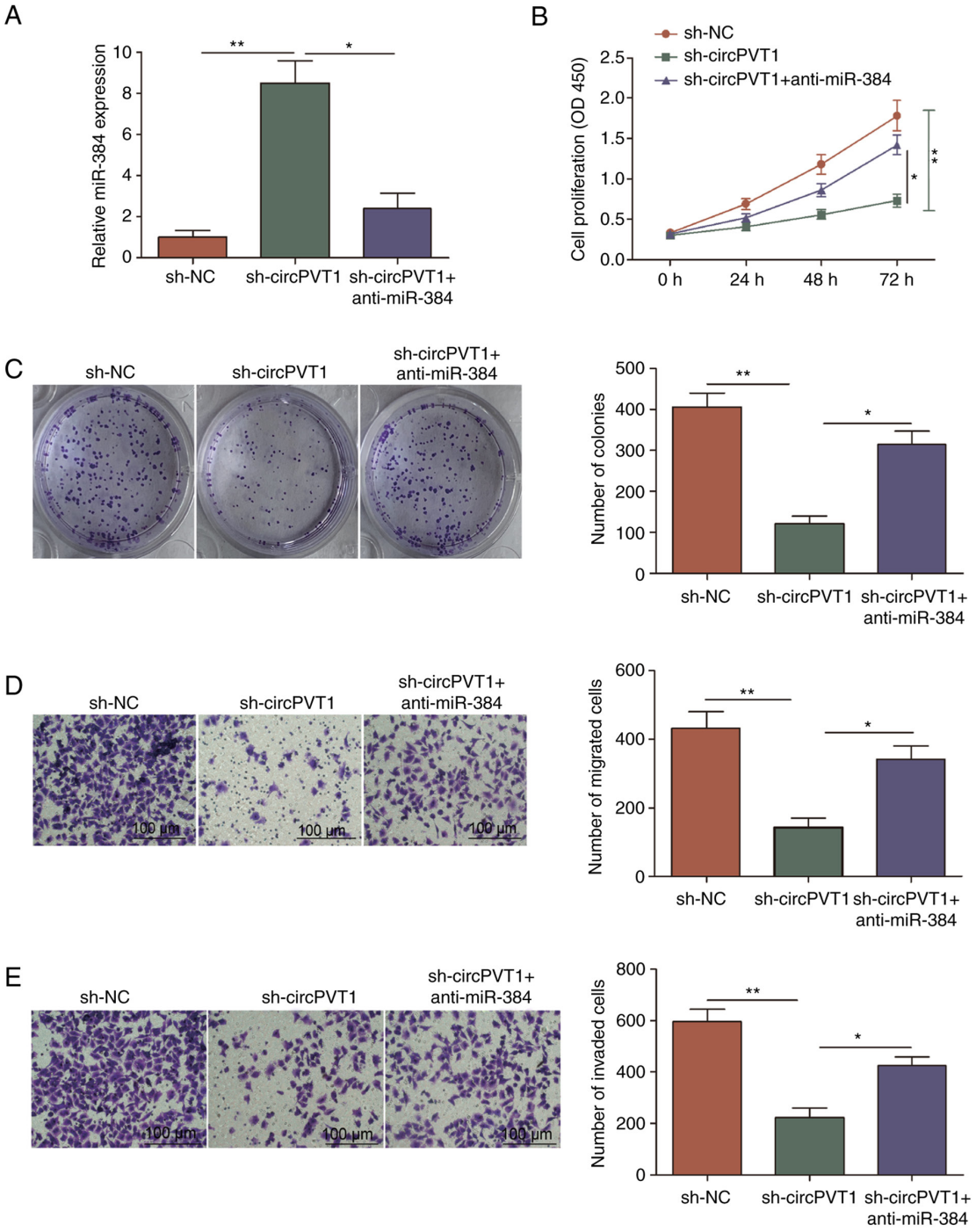

Figure 5. circPVT1 affects thyroid cancer cell proliferation and invasion by sponging miR-384. (A) Expression of miR-384 detected in TPC-1 cells transfected with sh-NC, sh-circPVT1 and sh-circPVT1 + anti-miR-384. (B) Proliferation, (C) colony formation, (D) migration and (E) invasion of TPC-1 cells transfected with sh-NC, sh-circPVT1 and sh-circPVT1 + anti-miR-384. Data are presented as the mean ± standard deviation. All experiments were conducted in triplicate. *P<0.05 and **P<0.01 vs. sh-NC. circPVT1, plasmacytoma variant translocation 1 circular RNA; sh, short harpin; NC, negative control; miR, microRNA; anti-miR, miR inhibitor.

circPVT1-knockdown decreased cell proliferation, migration and invasion. The present results suggested that circPVT1 functions as an oncogenic circRNA in thyroid cancer.

The majority of circRNAs localized in the cytoplasm exert their biological role by sponging miRs (31). The circRNA-miR regulation network is reported to be involved in tumorigenesis (32). Here, RT-qPCR assay showed that circPVT1 was primarily distributed in the cytoplasmic fraction of thyroid cancer cells, suggesting that circPVT1 may be involve in thyroid cancer progression by sponging miRs. Dual-luciferase reporter and RNA pull-down assay demonstrated that miR-384 is a target of circPVT1. Studies have demonstrated that miR-384 serves a tumor suppressive role in colorectal (24), non-small cell lung (25) and breast cancer (26). In thyroid cancer, relative expression of miR-384 is decreased and miR-384 serves as tumor suppressor by targeting protein kinase cAMP-activated catalytic subunit β (33). In the present study, miR-384

expression was downregulated in thyroid cancer tissue, which was consistent with previous results (33,34). Moreover, expression of circPVT1 was inversely correlated with miR-384 expression in thyroid cancer specimens. miR-384 inhibitor partially reversed the inhibitory effect mediated by circPVT1 depletion in thyroid cancer cells. Therefore, the circPVT1-miR-384 regulation axis may be a regulatory mechanism for thyroid cancer.

The present study showed that circPVT1 was upregulated in thyroid cancer tissue and was associated with poor prognosis of patients with thyroid cancer. Silencing of circPVT1 inhibited the proliferation, migration and invasion of thyroid cancer cells by regulating miR-384. These findings reveal a key role of the circPVT1/miR-384 axis in thyroid cancer progression, offering a potential novel therapeutic target for thyroid cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TH designed the study, performed experiments and wrote the manuscript. YL analyzed data. All authors have read and approved the final manuscript. TH and YL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All experimental protocols were approved by the Ethics Committee of Ningbo Medical Centre Lihuili Hospital, Ningbo, China (approval no. KY2022SL277-01). All participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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