CircPTPRM accelerates malignancy of papillary thyroid cancer via miR-885-5p/DNMT3A axis

Daping Chen1 | Xijiao Jiang2 | Huizhou Luo3 | Qingquan Hua4 | Farong Zhang2

1Department of Thyroid and Breast Surgery, Xianning Central Hospital, The First Affiliated Hospital of Hubei University of Science and Technology, Xianning, Hubei Province, China
2Department of Otorhinolaryngology, The First People’s Hospital of Jiangxia District, Xiehe Jiangnan Hospital, Wuhan, Hubei Province, China
3Department of Otorhinolaryngology, Liyuan Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei Province, China
4Department of Otolaryngology-Head and Neck Surgery, Renmin Hospital of Wuhan University, Wuhan, Hubei Province, China

Correspondence
Daping Chen, Department of Thyroid and Breast Surgery, Xianning Central Hospital, The First Affiliated Hospital of Hubei University of Science and Technology, No.228 Jingui Road, Xian’an District, Xianning 437000, Hubei Province, China. Email: dapingchen123@163.com

Farong Zhang, Department of Otorhinolaryngology, The First People’s Hospital of Jiangxia District, Xiehe Jiangnan Hospital, No.1 Wenhua Avenue, Zhifang Street, Jiangxia District, Wuhan 430200, Hubei Province, China. Email: 122113409@qq.com

Abstract
Background: Circular RNAs (circRNAs) are implicated in carcinogenesis, including papillary thyroid cancer (PTC). Despite of previous reports regarding the high expression of circPTPRM in PTC, the role and regulatory mechanism remain to be investigated.

Methods: CircPTPRM and miR-885-5p expression were examined, and the effects on cell proliferation, migration, and invasion were also measured. Immunoblotting was performed to evaluate DNA methyltransferase 3A (DNMT3A) and the epithelial-mesenchymal transition (EMT)-associated proteins.

Results: CircPTPRM was overexpressed in PTC tissues and cell lines, which predicted poor prognosis. CircPTPRM inhibition significantly alleviated the proliferation, migration, and invasion abilities. It was subsequently confirmed that circPTPRM competed with miR-885-5p for DNMT3A binding. CircPTPRM promoted PTC progression via miR-885-5p/DNMT3A signal axis.

Conclusion: Our data elucidated that circPTPRM may play an oncogenic role in PTC through circPTPRM/miR-885-5p/DNMT3A axis

KEYWORDS circular RNA, DNMT3A, miR-885-5p, papillary thyroid cancer

1 | INTRODUCTION

Thyroid cancer is one of the most common malignant tumors of the endocrine system,1,2 and papillary thyroid cancer (PTC) is the most common pathologic type.3 Thyroidectomy is one of the main treatment methods for PTC.4 Although the prognosis of patients with PTC is considerably satisfactory after surgical excision, the recurrence remains high due to distant metastasis.5,6 PTC not only brings physical trauma to patients with surgical treatment, but also brings heavy psychological burden due to the fear of disease recurrence. Therefore, it is of great value to conduct relevant basic research and clarify the molecular mechanism of the occurrence and development of PTC.

Circular RNAs (circRNAs) are widely involved in the progression of diverse malignancies.7 Plentiful circRNAs have demonstrated
regulate multiple biological processes of PTC cells, such as survival, apoptosis, invasion, and metastasis. Increasing studies report that circRNA is a crucial member of competitive endogenous RNA (ceRNA), and acts as a miRNA “sponge” to relieve the inhibitory effect on its target genes. Lv et al. have used RNA deep sequencing to determine the expression patterns of circRNAs in PTC tumors and discovered that circPTPRM is highly expressed in PTC patients. However, the precise roles of circPTPRM in the development of PTC and its associated ceRNA network remain to be further clarified.

2 | MATERIALS AND METHODS

2.1 | Patients and tissues

The experimental procedures were approved by the Ethics Committee of Xianning Central Hospital, The First Affiliated Hospital of Hubei University of Science and Technology. Paired tumor and paracancerous normal tissues were obtained from 50 patients who were diagnosed with PTC and signed informed consent.

2.2 | Cell lines

Human thyroid cell line Nthy-ori 3-1 and PTC cell lines were all obtained from the Chinese Academy of Sciences (Shanghai, China). Cell culture was performed at 37°C in DMEM medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin.

2.3 | Cell transfection

When 70%–80% confluence was achieved, cell transfection was performed with siRNA targeting circPTPRM (siRNA#1; sense 5’-GUAA UGAAGAAAGUCCACTT-3’ and antisense 5’-UGGGACUUUUC UUCAUUACTT-3’; siRNA#2; sense 5’-GAAAAGUCCAGGUGCU- TT-3’ and antisense 5’-ACAGCAUCGGACUUUUCTT-3’; 100 nM), miR-885-5p mimic (5’-UCCAUUACAUCCUCGCU-3’; 50 nM), miR-NC (5’-UUCUCGAACGGUCAGUGCUTT-3’; 50 nM), miR-885-5p inhibitor (5’-AGAGCCAGGGUAGUAUGGA-3’; 100 nM), inhibitor NC (5’-CAGUCUUUUGAGUGUACAA-3’; 100 nM), pcDNA3.1-based DNMT3A overexpression vector (100 nM) or corresponding negative controls (100 nM), purchased from Genepharma (Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

2.4 | Quantitative real-time PCR and RNase R digestion

Tissues and cultured cells were used for total RNA extraction using Trizol reagent (Invitrogen). SYBR Green RT-qPCR kit (Takara, Shiga, Japan) was applied for RT-qPCR reactions. Total RNAs of TPC-1 and KAT-5 cells were incubated for 30 min with 3 U/mg of RNase R or mock at 37°C. The relative levels of circPTPRM and PTPRM mRNA were quantified by qRT-PCR analysis. The following sequences of the primers were used for qPCR (5’-3’): circPTPRM forward: GGCGCATCTGCTGTTCTGTA; reverse: TTCACTTGGAACACAGACCTG; PTPRM forward: GGCGAGACGTTCTCAGGTG; reverse: AGAAGTCGGTTAGTAGAATCAG; miR-885-5p forward: GGGGTTCACGCTGACTGCT; reverse: CAGACAGAGAGGAGAGACGAC.

2.5 | Luciferase reporter assay and RNA immunoprecipitation (RIP) assay

The wild or mutated circPTPRM or DNMT3A luciferase plasmids were co-transfected with either the miR-885-5p mimic or miR-NC. After 48h transfection, the luciferase activity (Promega, Madison, Wisconsin, USA) was detected. RIP assay was performed using Imprint® RNA Immunoprecipitation Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the instructions. The cell extracts of TPC-1 and KAT-5 cells were incubated with RIP buffer containing magnetic beads coated with Ago2 (#2897; 1:50 dilution) or IgG (#8726; 1:20 dilution) antibodies (all from Cell Signaling Technology, Boston, MA, USA). After that, the co-precipitated RNAs were subjected to qRT-PCR analysis for the enrichments of circPTPRM and miR-885-5p.

2.6 | Cell viability assay

Cell viability was examined with the CCK-8 kit (Beyotime Biotechnology, Shanghai, China). At different time points (0 h, 24 h, 48 h, and 72 h), cultured cells (2000 cells/well) were mixed with 10 μl CCK-8 solution in a 96-well plate. After 4 h incubation, absorbance was analyzed at 450 nm using microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.7 | Colony formation assay

Cells were plated at 300 cells/well in 6-well plates and incubated for 15 days when colonies were visible. Crystal violet staining was performed and the number of colonies was counted.

2.8 | Flow cytometry

Cells were collected and suspended in the binding buffer (BD Biosciences, Franklin, NJ, USA). Subsequently, Annexin V-fluorescein
isothiocyanate (Annexin V-FITC; BD Biosciences) and propidium iodo
de (PI; BD Biosciences) were added to simultaneously incubate with
PTC cells in the dark. The apoptosis rate was analyzed by the
FACS Cantoll flow cytometer (BD Biosciences).

2.9 | Wound healing assay

Cells were seeded in 6-well plates at a density of 4 × 10^5 cells/well.
Once the cells reached 90% confluence, a wound area was carefully
created by scraping the cell monolayer with a sterile 200 μl pipette
tip, from one end to the other end of the well. The detached cells
were removed by washing with PBS. Cells migrated to the wounded
region were observed by Olympus inverted microscope and photo-
graphed (100x magnification) at 0 h and 24 h.

2.10 | Transwell assays

Transwell chamber (BD Biosciences, Bedford, MA, USA) with or
without Matrigel coating was used to evaluate cell migration and
invasion. Serum-free media with cultured cells was added into the
upper wells, while the lower chambers were filled with conditioned
media with 20% FBS. The migrated or invaded cells were fixed and
stained with 0.5% crystal violet and representative images were
captured under microscopy at 200x magnification.

2.11 | Western blotting

TPC-1 and KAT-5 cells were dissolved in RIPA buffer (Beyotime
Biotechnology) containing protease inhibitor for the extraction of
total protein, and the protein quantification was then measured by
BCA Protein Assay Kit (Beyotime Biotechnology). Proteins (50 μg)
were exposed to 10% SDS-PAGE and electrophoretically on a PVDF
membrane (EMD Millipore, Bedford, MA, USA). The membranes
were incubated overnight at 4°C with indicated primary antibod-
ies (1:1000 dilution; all from Cell Signaling Technology): DNMT3A
(#5741), and GAPDH (#5174). Secondary antibodies (#7074) di-
luted at 1:1000 were subsequently used for further incubation
and visualized using the enhanced chemiluminescence method (Beyotime
Biotechnology).

2.12 | Statistical analysis

All the experiments were performed at least in triplicate, and
at least repeated for three times. SPSS 19.0 statistical software
(IBM, Chicago, IL, USA) was used to analyze the data presented
as mean ± standard deviation (SD). Student’s t-test or analysis of
variance followed by Tukey’s test was used to analyze the results.

Kaplan–Meier survival analysis was used to estimate the associa-
tion between circPTPRM expression and survival outcomes of our
patients, and differences were estimated by the log-rank test. The
correlation analysis was determined using Pearson’s correlation test.
Statistical significance was set at p < 0.05.

3 | RESULTS

3.1 | CircPTPRM is overexpressed in PTC tissues
and cells

Of note, the relative level of circPTPRM was significantly increased
in PTC tissues as compared to the normal tissues (Figure 1A).
Consistently, circPTPRM was also upregulated in several human PTC
Tissue lines (Figure 1B). As shown in Figure 1C, circPTPRM was resis-
tant to RNase R, whereas PTPRM mRNA was degraded by RNase R
digestion, revealing the stability of circPTPRM. As demonstrated by
Kaplan–Meier analysis, PTC patients with high circPTPRM expres-
sion had a poor overall survival (Figure 1D).

3.2 | CircPTPRM knockdown restrains malignant
phenotypes of PTC cells

CircPTPRM knockdown cell lines (TPC-1 and KAT-5 cells) were
constructed to further analyze the oncogenic properties of circPT-
PRM. The downregulation efficiency of circPTPRM was confirmed
via qRT-PCR in these two cells, which showed the highest knock-
down efficiency of siRNA#1 (Figure 2A). CCK-8 assay showed that
circPTPRM deficiency inhibited the cell viability of TPC-1 and KAT-5
cells (Figure 2B). Colony formation assay revealed that circPTPRM
silencing markedly suppressed the colony formation ability of TPC-1
and KAT-5 cells (Figure 2C). Flow cytometry analyses found that
circPTPRM depletion contributed to significant acceleration of ap-
optosis rate in TPC-1 and KAT-5 cells (Figure 2D). Wound healing and
Transwell assays uncovered that circPTPRM interference reduced
the abilities of migration (Figure 2E,F) and invasion (Figure 2G) which
was also evidenced by increased level of E-cadherin and downregu-
lated expressions of N-cadherin and Vimentin (Figure 2H,I). The
changes of EMT markers were consistent with morphological altera-
tions. As shown in Figure 2J, circPTPRM-silencing cells displayed
cobblestone-like cell morphology, the typical features of epithelial
cells, compared with the control cells which exhibited a mesenchy-
mal phenotype with elongated shape and reduced cell–cell contact
observed by inverted microscopy.

3.3 | MiR-885-5p is downregulated in PTC and
sponged by circPTPRM

Furthermore, predicted by CircInteractome (https://circi ntera
tome.nia.nih.gov), circPTPRM might potentially interact with
miR-885-5p through complementary base pairing (Figure 3A). The dual-luciferase reporter assay showed that the luciferase activity of circPTPRM wild plasmids was notably restrained by up-regulating miR-885-5p (Figure 3B). MiR-885-5p upregulation was achieved through the transfection of the miRNA mimic (Figure 3C). Additionally, the interaction of circPTPRM with miR-885-5p was verified by RNA pull-down assay (Figure 3D). Figure 3E showed that miR-885-5p was upregulated by circPTPRM silencing. The down-regulation of miR-885-5p was observed in tumor tissues (Figure 3F), and a negative association was shown between it and circPTPRM expression (Figure 3G).

### 3.4 | CircPTPRM regulates DNMT3A expression by targeting miR-885-5p

The schematic illustration of miR-885-5p binding sites in DNMT3A 3’UTR was predicted by starBase V3.0 (https://starbase.sysu.edu.cn/; Figure 4A). The results of luciferase assay illustrated that miR-885-5p mimic led to the reduction of the luciferase activities of wt-DNMT3A 3’-UTR (Figure 4B). The DNMT3A mRNA and protein levels were declined upon miR-885-5p overexpression (Figure 4C,D). As demonstrated by qRT-PCR, miR-885-5p could be silenced by the inhibitor of miR-885-5p (Figure 4E). DNMT3A expression at protein levels was inhibited by circPTPRM knockdown, which was restored by miR-885-5p inhibition (Figure 4F). The upregulated DNMT3A level was observed in PTC tissues (Figure 4G). DNMT3A mRNA was positively correlated with circPTPRM expression (Figure 4H), while negatively correlating with miR-885-5p expression in PTC tissues (Figure 4I).

### 3.5 | CircPTPRM aggravates PTC cell malignant phenotypes by regulating miR-885-5p/DNMT3A signaling axis

DNMT3A was successfully overexpressed using the overexpression vector, as confirmed by western blotting (Figure 5A). The down-regulation of circPTPRM resulted in the knockdown of DNMT3A protein levels, while DNMT3A overexpression overturned this effect (Figure 5B). Moreover, circPTPRM siRNA-induced suppressive effects on cell viability (Figure 5C), migratory ability (Figure 5D) and invasive capability (Figure 5E) could be effectively relieved by the enforced expression of DNMT3A. Besides, DNMT3A overexpression abrogated the impact of circPTPRM siRNA on the changes of EMT markers (Figure 5F,G).
The implication of circRNAs has been clarified in the development of diverse malignancies, including PTC. For instance, circ_0039411 is overexpressed in PTC tissues, and circ_0039411 decoys miR-423-5p, upregulates the expression of SOX4, and promotes CRC cell proliferation, migration, invasion, and glycolysis. Circ_0067934 expression is augmented in PTC tissues, and

**FIGURE 2** CircPTPRM knockdown restrains malignant phenotypes of PTC cells. (A) After circPTPRM inhibition in TPC-1 and KAT-5 cells, the transfection efficiency was analyzed via qRT-PCR (A); cell viability was determined by CCK-8 assay (B) and colony formation assay (C); cell apoptosis was analyzed by flow cytometry (D); cell migration and invasion capabilities were evaluated by wound healing (E) and Transwell assays (F,G); the protein levels of E-cadherin, N-cadherin and Vimentin were determined by western blot (H,I). (J) Micrographs of si-NC-transfected cells and circPTPRM-silencing cells by light microscope (100× magnification). Data are expressed as the mean±SD. **p<0.01; ***p<0.001 considered as significant.

4 | DISCUSSION

The implication of circRNAs has been clarified in the development of diverse malignancies, including PTC. For instance,
circ_0067934 works as an oncogene in CRC by modulating miR-1301-3p/HMGB1 axis.\textsuperscript{15} CircNRIP1 expedites the proliferation, invasion, and tumor growth in PTC by sponging miR-195-5p and regulating p38 MAPK and JAK/STAT signaling pathways.\textsuperscript{16} Luo et al.\textsuperscript{17} found aberrant overexpression of circPTPRM in HCC tissues and cells, and its expression was associated with the pathological metastasis, recurrence, and survival; circPTPRM may function as an oncogene during the tumorigenesis of HCC. However, there are few reports on the role of circPTPRM in other malignant tumors. Corresponding to the previous study,\textsuperscript{13} circPTPRM was uncovered to be an overexpressed circRNA in PTC. In our work, high expression of circPTPRM indicated the unfavorable prognosis. Additionally, functional assays revealed that circPTPRM silencing impeded PTC cell malignant phenotypes. These findings suggest that circPTPRM exerted oncogenic properties in PTC, which might be a potential therapeutic target for PTC.

Multiple miRNAs have been reported to participate in PTC progression. For example, miR-181a was upregulated in PTC tissues, which could upregulate S100A2 expression by targeting KDM5C to facilitate PTC progression.\textsuperscript{18} MiR-613 was underexpressed in PTC tissues and cells, and the inhibition of PTC progression was associated with miR-613 overexpression by directly targeting TAGLN2.\textsuperscript{19} In this work, circPTPRM was validated to have binding sites with miR-885-5p. Given that circRNAs can act as miRNA sponges to promote the development of cancers,\textsuperscript{20–22} we presumed that circPTPRM might contribute to PTC progression by interacting with miR-885-5p that has been reported to restrain the progression of hepatocellular carcinoma (HCC),\textsuperscript{23} cholangiocarcinoma,\textsuperscript{24} and gastric cancer.\textsuperscript{25} The anti-tumor role of miR-885-5p was also verified in PTC development.\textsuperscript{26} Further functional studies showed that circPTPRM serves as a miR-885-5p sponge to promote PTC progression.
DNMT3A is a widely reported DNA methyltransferase and plays a pivotal role in DNA methylation modification and epigenetic regulation. In recent years, studies have shown that DNMT3A is ubiquitously underexpressed in various normal tissues, but abnormally upregulated in various tumor tissues, including PTC tumor tissues, participating in the malignant process of PTC. In the current work, DNMT3A was identified the direct target of miR-885-5p, and their expression was negatively correlated in PTC tissues. Furthermore, a novel integrated circPTPRM/miR-885-5p pathway showed the regulatory effects on DNMT3A. Meanwhile,
circPTPRM may aggravate PTC carcinogenesis via the regulation of miR-885-5p/DNMT3A signal axis.

Taken together, we concluded that circPTPRM may exert as a novel oncogenic circRNA in PTC cells in vitro by regulating the miR-885-5p/DNMT3A pathway and thus affecting the proliferation, migration, and invasion. However, the lack of larger cohort is one of the limitation of the present study. Furthermore, the in vivo pro-tumor effect of circPTPRM deserves further investigation.
CONFLICT OF INTEREST
The authors state that there are no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT
All data generated or analyzed during this study are included in this published article.

ORCID
Doping Chen https://orcid.org/0000-0001-8098-4756

REFERENCES
1. Seib CD, Sosa JA. Evolving understanding of the epidemiology of thyroid cancer. Endocr Metab Clin North Am. 2019;48(1):23-35.
2. Araque KA, Gubbi S, Klubo-Gwiazdinska J. Updates on the management of thyroid cancer. Horm Metab Res. 2020;52(8):562-577.
3. Abdullah MI, Junit SM, Ng KL, Jayapalan JJ, Karikalan B, Hashim OH. Papillary thyroid cancer: genetic alterations and molecular biomarker investigations. Int J Med Sci. 2019;16(3):450-460.
4. Diez JJ, Anda E, Sastre J, et al. Recovery of parathyroid function in patients with thyroid cancer treated by total thyroidectomy: an analysis of 685 patients with hypoparathyroidism at discharge of surgery. Endocrinol Diabetes Nutr (Engl ed). 2021;68(6):398-407.
5. Dedivitis RA, Matos LL, Guimarães AV, Castro MAF, Petrarolha SP. Circular RNAs in human papillary thyroid carcinoma based on RNA deep sequencing. Int J Biol Markers. 2021;36(6):1866-1877.
6. Luo Z, Mao X, Cui W. Circular RNA expression and circPTPRM promotes proliferation and migration in hepatocellular carcinoma. Med Oncol. 2019;36(10):86.
7. Wang Y, Ye H, Yang Y, Li J, Cen A, Zhao L. miRNA-181a promotes the oncogene S100A2 and enhances papillary thyroid carcinoma growth by mediating the expression of histone demethylase KDM5C. J Endocrinol Invest. 2021;45:17-28.
8. Chu J, Tao L, Yao T, et al. Circular RNA circRUNX1 promotes thyroid cancer via enhancing MAPK1 expression by serving as the sponge miR-1301-3p and regulating DDHD2 expression. Cell Signal. 2021;88:110155.
9. Huang Y, Zhang H, Wang L, et al. miR-613 inhibits the proliferation, migration, and invasion of papillary thyroid cancer cells by directly targeting TAKL2. Cancer Cell Int. 2021;21(1):494.
10. Chen W, Zhang T, Bai Y, et al. Upregulated circRAD18 promotes tumor progression by reprogramming glucose metabolism in papillary thyroid cancer. Gland Surg. 2021;10(8):2500-2510.
11. Zhang Y, Zhang Y, Feng Y, et al. Construction of circRNA-based ceRNA network and its prognosis-associated subnet of clear cell renal cell carcinoma. Cancer Med. 2021;10:8210-8221.
12. Lixin S, Wei S, Haibin S, Qingfu L, Tiemin P. miR-885-5p inhibits proliferation and metastasis in gastric cancer by targeting miR-335-5p sponge. Cell Signal. 2021;88:110155.
13. Li C, Wang X, Song Q. MicroRNA 885-5p inhibits hepatocellular carcinoma metastasis by repressing AEG1. Onco Targets Ther. 2020;13:981-988.
14. Deng J, Chen D, Jiang X, Luo H, Hua Q. Circular RNA circ0039411 promotes papillary thyroid carcinoma progression by sponging mir-195-5p and modulating the P38 MAPK and JAK/STAT pathways. Diagn Pathol. 2021;16(1):93.
15. Luo Z, Mao X, Cui W. Circular RNA expression and circPTPRM promotes proliferation and migration in hepatocellular carcinoma. Med Oncol. 2019;36(10):86.
16. Li C, Zhu L, Fu L, et al. CircRNA NRIP1 promotes papillary thyroid carcinoma progression by sponging mir-195-5p and modulating the P38 MAPK and JAK/STAT pathways. Diagn Pathol. 2021;16(1):93.

How to cite this article: Chen D, Jiang X, Luo H, Hua Q. Zhang F. CircPTPRM accelerates malignancy of papillary thyroid cancer via miR-885-5p/DNMT3A axis. J Clin Lab Anal. 2022;36:e24688. doi: 10.1002/jcla.24688