Silencing circRNA protein kinase C iota (circ-PRKCI) suppresses cell progression and glycolysis of human papillary thyroid cancer through circ-PRKCI/miR-335/E2F3 ceRNA axis

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Abstract. The circular RNA PRKCI (circ-PRKCI; ID: hsa_circ_0122683) is highly expressed in human papillary thyroid cancer (PTC) tumors according to GSE93522 dataset. However, its role in PTC tumorigenesis remains to be documented. Here, quantitative real-time PCR showed that expression of circ-PRKCI was abnormally upregulated in human PTC patients’ tumors and cells, and higher circ-PRKCI might predict lymph node metastasis and recurrence. Functionally, cell behaviors were measured by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay, colony formation assay, fluorescence-activated cell sorting method, scratch wound assay, transwell assay, western blotting, and assay kits for glucose and lactate. As a result, circ-PRKCI knockdown could suppress cell cycle progression of PTC cells and restrain the abilities of cell proliferation, colony formation, wound closure, invasion, glucose consumption and lactate production, accompanied with decreased levels of matrix metalloproteinase-2 (MMP2), MMP9 and Snail. Moreover, above-mentioned inhibition could be imitated by overexpressing microRNA-335-5p (miR-335). Molecularly, circ-PRKCI functioned as a sponge for miR-335 and miR-335 could further targeted E2F transcription factor-3 (E2F3), according to dual-luciferase reporter assay and RNA immunoprecipitation. However, downregulating miR-335 diminished the effects of circ-PRKCI role on cell growth, metastasis and glycolysis in PTC cells; besides, there was a counteractive effect between miR-335 upregulation and E2F3 upregulation in PTC cells as well. Furthermore, xenograft experiment revealed that silencing circ-PRKCI could retard tumor growth of PTC cells in vivo. Collectively, circ-PRKCI exerted oncogenic role in PTC by antagonizing cell progression and glycolysis via regulating miR-335/E2F3 axis, suggesting circ-PRKCI was a potential biomarker and target for PTC.

Key words: circRNA protein kinase C iota, microRNA-335, E2F transcription factor-3, Papillary thyroid cancer

PAPILLARY THYROID CARCINOMA (PTC) accounted for approximately more than 85% of thyroid cancers that are the most common endocrine malignancies [1]. Originating from the follicular epithelium, PTC comprises well-differentiated thyroid epithelial neoplasms [2]. The incidence of PTC is on the rise and recurrence happens in more than 25% PTC patients [3]. It has been well-ingrained that genetic changes, epigenetic alterations and environmental factors are the basis for carcinogenesis of cancers including thyroid cancer [4].

RNA circularization is a pervasive feature of cancer cells, during which circular RNAs (circRNAs) are generated [5]. CircRNAs are a class of circular transcripts with a range of protein-coding and non-coding functions [6]. Notably, the covalently closed loop endows circRNAs...
peculiarities, such as resistance to ribonuclease R (RNase R) digestion, evolutionary conservation and tissue specificity, as well as the long half-life [7]. CircRNAs are differently expressed in PTC patients, and dysregulation of circRNAs is a new hot topic in the repertoire of molecular alterations in PTC. Landscape of circRNA expression profiles have been revealed in PTC tissues [8-10]. Protein kinase C iota (PRKCI) gene is frequently amplified in several human cancers [11], and circRNA PRKCI (circ-PRKCI; ID: hsa_circ_0122683) is highly expressed in PTC via Gene Expression Omnibus (GEO) database (accession: GSE93522) [12]. However, the aberrant functions of circ-PRKCI remain unclear yet.

According to GSE102686, a circRNAs-associated competitive endogenous RNAs (ceRNAs) network in PTC has been demonstrated [13], and circRNAs could function as sponges for microRNAs (miRNAs) at transcription level to modulate endogenous messenger RNAs (mRNAs) expression [14]. E2F transcription factor-3 (E2F3) is the key downstream target of Retinoblastoma protein (pRb), a tumor suppressor [15]. Furthermore, there is a complex reciprocal regulation network between E2F3 and miRNAs, and multiple miRNAs could guide E2F3 via target binding [16]. miR-335-5p (miR-335) acts as oncogene or tumor suppressor in diverse tumors [17], and it is stated that miR-335 might play anti-tumor role in PTC [18].

Except for transcriptomes [19], proteomes and metabolomics have also been deciphered to be novel biomarkers for PTC [20]. Metabolic changes are closely related to PTC pathogenesis, and PTC is characterized with increased glycolysis [21]. Therefore, the aim of this study was to investigate the expression and functional role of circ-PRKCI in malignant cell progression of PTC and glycolysis, as well as the relationship among circ-PRKCI, miR-335 and E2F3.

Materials and Methods

Patients and cells

A sum of 48 patients with PTC were collected from Guizhou Provincial People’s Hospital, and the PTC tumor and corresponding non-tumor tissues were collected during thyroidectomy. The sampling and experimental processes were performed with the approval of the Ethics Committee of Guizhou Provincial People’s Hospital. The PTC patients were pathologically confirmed either intra- or post-operatively, and without other malignancies; no patients received any antitumor treatment prior to this surgery. Tissue collection was performed after we obtained the informed written consent from each patient. Clinicopathological factors of these PTC patients were summarized in Table 1, and these patients were divided into two groups according to the circ-PRKCI expression: High group (n = 24) was that circ-PRKCI level was higher than the median, and Low group (n = 24) was that circ-PRKCI level was lower than the median. The 8th edition of the American Joint Committee on Cancer classification system was used for tumor-node metastasis (TNM) staging. The tissues were stored in liquid nitrogen immediately for further RNA and protein extraction.

Human normal thyroid follicular epithelial cells (Nthy-ori 3-1; #90011609) and three human PTC cells including PTC-1 (#0397), IHH-4 (#1079) and SNU-790 (#00790) were purchased from European Collection of Authenticated Cell Cultures (ECACC; Salisbury, UK), Banco de Celulas do Rio de Janeiro (BCRJ; Duque de Caxias, RJ, Brazil), Japanese Collection of Research Bioresources Cell Bank (JCRB; Osaka, Japan), and Korean Cell Line Bank (KCLB; Seoul, Korea), respectively. These cells were cultured in RPMI-1640 media (HyClone, Logan, UT, USA) with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (FBS; Hyclone) in a humidified atmosphere with 5% CO₂ at 37°C.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNAs in tissues and cells were extracted with TRIzol reagent (Beyotime, Shanghai, China) in accordance with the manufacturer’s manual. RNAs in cytoplasm and nucleus were separated via Cytoplasmic & Nuclear RNA Purification Kit (Norgen Biotek, Thorold, ON, Canada) according to the operating instructions. RNA quality and quantity were evaluated by electrophoresis in agarose and NanoDrop OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). 2 μg of extracted total RNAs from TPC-1 and IHH-4 cells were treated with 3 U RNase R (Generese Biotech, Guangzhou, China) at 37°C for 30 min. For Actinomycin D (Act D) assay, Act D (R&D SYSTEMS, Minneapolis, MN, USA) was purchased and dissolved in dimethylsulfoxide (DMSO) and the stock concentration of Act D was 50 mM. TPC-1 and IHH-4 cells were exposed to 3 μM Act D (R&D SYSTEMS) or 0.1% DMSO for 4, 8, and 12 h, followed with total RNAs isolation.

Above-mentioned RNAs were subjected to cDNA synthesis using HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China), and different cDNAs were amplified and quantified using ChamQ SYBR Color qPCR Master Mix (Vazyme) and corresponding primers. Relative expression levels of circ-PRKCI, PRKCI, miR-335 and E2F3 were normalized to U6 (for miRNA) or β-actin (for other RNAs) using 2^{ΔΔCt} method. Each RT-qPCR reaction was repeated at least
4 wells. The primer sequences were circ-PRKCI, 5’-CCAGATGACGAGTGGTTCTGATGCTGGT-3’ (forward primer, F) and 5’-CTCTCTGGTTCTGAGGTGTTG-3’ (reverse primer, R); PRKCI, 5’- GTGTTGACTGGTGGGCTCTT-3’ (F) and 5’- CCTCTGTGTTCTGGTCAGGG-3’ (R); miR-335, 5’- TCAAGAGCAATAACGAAAAATGT-3’ (F) and 5’- GCGAGCACAGAATTAATACGAC-3’ (R); E2F3, 5’- TTGGAAACTCCGACTGCAAAT-3’ (F) and 5’-TTTCTCATCTCTCGCTCCTG-3’ (R); U6, 5’- CTCGCTTCGGCAGCACA-3’ (F) and 5’- AACGCTTCACGAAATTTGCGT-3’ (R); β-actin, 5’- GACCTCTATGCCAACAGTGC-3’ (F) and 5’- GTACTCCTGCTTGCTGACTCCAC-3’ (R).

Cell transfection

TPC-1 and IHH-4 cells were re-seeded in 6-well plate for 24 h and transfected with oligonucleotides and vectors using Exfect 2000 Transfection Reagent (Vazyme) according to the manufacturer’s instructions. The oligonucleotides were obtained from Sangon Biotech (Shanghai, China) and included siRNAs against circ-PRKCI (si-circ-PRKCI#1, 5’-UCAACACUCAGAAUCUCGUCAU C-3’ and si-circ-PRKCI#2, 5’-UGAACAUCUGUACUCGAGGUGGCAGG-3’), miR-335 mimic (5’-UCAAGAGCAAAU ACGAAUAUGGU-3’), and miR-335 inhibitor (5’-ACA UUUUCGUUAUUGCUUCUAU-3’), as well as their negative controls (si-NC, 5’- UCUUUAGGGGUGUGCGGUAA-3’; NC mimic, 5’-GGUUCGUACGUACACUGU-3’; and NC inhibitor, 5’- UGAACAGUGUACGUA CGAAC-3’). The vectors included circ-PRKCI overexpression vector, E2F3 overexpression vector, circ-PRKCI silencing vectors (pGreenPuro; YouBio, Changsha, China) expressing shRNA against circ-PRKCI (sh-circ-PRKCI, 5’-GATGACGAGTGGTTCTGATGCTGGT-3’ and 5’-TCAAGAGCAATAACGAAAAATGT-3’ (F) and 5’-TTTCTCATCTCTCGCTCCTG-3’ (R)), and dual-luciferase reporter vectors (pGL4; Promega, Madison, WI, USA) carrying wild type (wt) and mutated type (mut) of circ-PRKCI or 3’-untranslated region (E2F3 3’UTR). The full length of circ-PRKCI (http://www.circbank.cn/hsa_circ_0122683) and E2F3

| Parameters | Case | High (n = 24) | Low (n = 24) | p value |
|------------|------|--------------|--------------|---------|
| Age | | | | |
| ≤45 | 30 | 17 | 13 | 0.233 |
| >45 | 18 | 7 | 11 | |
| Gender | | | | |
| Male | 6 | 2 | 4 | 0.383 |
| Female | 42 | 22 | 20 | |
| Tumor Size | | | | |
| ≤1 cm | 13 | 5 | 8 | 0.330 |
| >1 cm | 35 | 19 | 16 | |
| TNM stage | | | | |
| I-II | 29 | 12 | 17 | 0.140 |
| III | 19 | 12 | 7 | |
| Extrathyroid extension | | | | |
| No | 44 | 23 | 21 | 0.296 |
| Yes | 4 | 1 | 3 | |
| Lymph node metastasis | | | | |
| No | 27 | 9 | 18 | 0.009* |
| Yes | 21 | 15 | 6 | |
| Recurrence | | | | |
| No | 44 | 20 | 24 | 0.037* |
| Yes | 4 | 4 | 0 | |

High circ-PRKCI in tumor tissues was significantly correlated with lymph node metastasis and recurrence in PTC patients, and was little associated with the age, gender, tumor size, TNM stage, and extrathyroid extension. Abbreviation: TNM, tumor node metastasis. *p < 0.05, Chi-square test.

Circ-PRKCI role in PTC
formed colonies and invaded cells were stained with 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay, colony formation assay and transwell assay

Transfected TPC-1 and IHH-4 cells were inoculated in 96-well plate at density of 5,000 cells per well and in 6-well plate with 100 cells/well. Every transfection group was set at least 3 paralleled wells. In brief, these cells were fixed in 4% paraformaldehyde at room temperature for 30 min. Eventually, colony formation Kit (Beyotime). In brief, these cells were fixed in RPMI-1640 media at established time points. After cell incubation at 37°C for 4 h, the MTT-containing supernatant was removed and 100 μL DMSO (Yeasen) was added into each well. The colorimetric analysis was measured at 490 nm on Multiskan Ascent 354 microplate reader (Bio-Tek, Instruments, Neufahrn, Germany).

Cells in 6-well plate were further cultivated for another 14 days with complete RPMI-1640 media refresh every three days for colony formation assay, and three repeat wells were included in each group. For invasion assay, transfected TPC-1 and IHH-4 cells (1 × 10⁶) were re-suspended in serum-free RPMI-1640 media and inoculated in the upper transwell chamber (Corning, Corning, NY, USA) pre-coated with Matrigel (Solarbio, Shanghai, China); the transwell chamber was located in 24-well plate, and the bottom chamber was filled with RPMI-1640 media containing 20% FBS. These cells in transwell assay were cultured for another 48 h. Later, formed colonies and invaded cells were stained with 0.5% crystal violet (Beyotime) at room temperature for 30 min following cell fixation by 4% paraformaldehyde at room temperature for 30 min. Eventually, colony counts and invaded cells were determined by microscope at 100× amplification.

Fluorescence-activated cell sorting (FACS) method

After transfection, TPC-1 and IHH-4 cells were collected and subjected to Cell Cycle and Apoptosis Analysis Kit (Beyotime). In brief, these cells were fixed in 70% ethanol at 4°C for 12 h, and then incubated in propidium iodide (PI) staining solution containing 10 μL RNase A (50×), 25 μL PI (20×), and 500 μL Binding Buffer at 37°C for 30 min in the dark. Cell cycle distribution was analyzed using FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo X 10.6.2 software (TreeStar, San Carlos, CA, USA).

Scratch wound assay

TPC-1 and IHH-4 cells with different transfection were transferred in 12-well plate in three repeat wells, and a small linear wound was created by scratching the cells from plate with 10 μL disinfected Eppendorf tip. Then, the remaining cells were cultured in serum-free RPMI-1640 media for another 24 h. The wound areas were photographed at 0 and 24 h under a microscope. Cell migration distance was analyzed by ImageJ 1.8.0 software (NIH, Bethesda, MD, USA) and wound closure rate was calculated.

Total protein extraction and western blotting

Total proteins were extracted from lysed tissues and cells using RIPA Lysis Buffer (Beyotime), and the concentration was determined by BCA Protein Assay Kit (Beyotime). 30 μg proteins were added with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Sample Loading Buffer (Beyotime) and boiled at 90°C for 8 min. Then, protein samples were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes, followed with membrane blocking with 5% non-fat milk at room temperature for 1 h. Then, the membranes carrying proteins were in sequence incubated with diverse primary antibodies (at 4°C for 8 h) and secondary antibodies (at room temperature for 1 h) prior to chemiluminescence using ECL chemiluminescence detection kit (Vazyme). The antibodies were purchased from Santa Cruz Biotechnology (Shanghai, China) and Beyotime (Shanghai, China): E2F3 (#sc-878, 1:1,000), matrix metalloproteinase-2 (MMP2; AF1420, 1:1,000), MMP3 (#AF7482, 1:1,000), Snail (#AF8013, 1:1,000), β-actin (#AF5003, 1:1,000), HRP-conjugated anti-rabbit IgG (#sc-2004, 1:5,000).

Glucose and lactate assay kits

Glucose Uptake Cell-Based Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) and Glycolysis Cell-Based Assay Kit (Cayman Chemical) were utilized to determine the levels of glucose consumption and lactate production in TPC-1 and IHH-4 cells with different transfection based on the protocols. Briefly, fluorescence was detected (excitation/emission = 485/535 nm) for glucose levels, and absorbance was measured (490 nm) for lactate levels. All data were obtained on SpectraMax M5e multimode microplate reader (Molecular Devices, San Jose, CA, USA).

Dual-luciferase reporter assay and RNA immunoprecipitation (RIP) assay

The dual-luciferase reporter system (Promega) was employed to confirm the target binding between miR-335 and either circ-PRKCI or E2F3 3’UTR. TPC-1
and IHH-4 cells were co-transfected with pGL4 vectors carrying wt-circ-PRKCI, mut-circ-PRKCI, wt-E2F3 3′UTR or mut-E2F3 3′UTR and miRNA mimics (miR-335 mimic or NC mimic) using Exfect 2000 Transfection Reagent (Vazyme). The transfected cells were examined of Firefly and Renilla luciferase activities post-transfection for 48 h. The relative luciferase activity was Firefly luciferase activity normalized to that of Renilla.

RIP assay was performed using Magna RIP™ RNA-binding protein immunoprecipitation kit (EMD Millipore, Billerica, MA, USA) in TPC-1 and IHH-4 cells with transfection of miR-335 mimic or NC mimic. Cell lysate was obtained in RNA Lysis Buffer, and then incubated with magnetic beads pre-conjugated with anti-Ago2 (EMD Millipore) or negative control anti-IgG (IgG; EMD Millipore) at 4°C overnight. After washed with Washing Buffer, immunoprecipitated RNAs were isolated using TRIzol reagent (Beyotime). The enriched levels of circ-PRKCI and E2F3 were analyzed by qPCR.

Xenograft experiments
A total of 10 BALB/c nude mice (female, 5-week-old) were purchased from Vital River Laboratory Animal Technology (Beijing, China) and housed in accordance with the Guide for the Care and Use of Laboratory Animals (GB/T35892-2018; Standardization Administration of the People Republic of China). This animal experiment was approved by the Animal Care and Use Committee of Guizhou Provincial People's Hospital. TPC-1 cells (1 × 10⁷ cells/mouse) were re-suspended in 150 μL of 1:1 PBS/Matrigel, and then were subcutaneously injected into posterior flanks of mice. After cell inoculation for 4 days, pGreenPuro vectors expressing sh-circ-PRKCI or sh-NC were injected into these mice (5 mice/group) every four days at the same position of cell inoculation. The mice were monitored every 4 days, and the length (L) and width (W) of tumors were monitored using digital caliper. Post-injection for 4 weeks, all mice were euthanized and tumors were dissected, weighted and stored. Tumor volume was calculated using the formula: 0.5 × (L × W²).

Statistical analysis
All the experiments were repeated three times, and data were shown as means ± standard deviation. The data were analyzed on GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA) and differences were estimated by Student’s t-test for 2 groups or one-way analysis of variance for ≥3 groups following Tukey’s post-hoc analysis. Pearson correlation coefficient analysis was used to determine the correlation among circ-PRKCI, miR-335 and E2F3 expression. Chi-square test analyzed the correlation between circ-PRKCI expression and clinicopathological factors of PTC patients. P < 0.05 represented statistical significance.

Results
circ-PRKCI was an upregulated circRNA in PTC patients and cells
According to qPCR data, expression of circ-PRKCI was higher in human PTC tumors and cells (TPC-1, IHH-4 and SNU-790) than normal thyroid tissues and the follicular epithelial Nthy-ori 3-1 cells (Fig. 1A and 1B). Since SNU-790 cells exhibited the lowest level of circ-PRKCI among these three PTC cells, we chose PTC-1 and IHH-4 cells for further functional experiments. To determine the authenticity of circular structure, RNase R and Act D treatments were carried out. As a result, comparing to its host PRKCI mRNA, circ-PRKCI expression was stable in RNase R-treated RNAs (Fig. 1C and 1D), and Act D-disposed TPC-1 and IHH-4 cells (Fig. 1E, 1F and Fig. S1). Moreover, the overwhelming majority of circ-PRKCI and β-actin was discovered to locate in the cytoplasm of TPC-1 and IHH-4 cells via nucleo-cytoplasmic separation, and U6 was almost distributed in the nucleus (Fig. 1G and 1H). These data demonstrated that circ-PRKCI was a cytoplasmic circRNA that was upregulated in PTC cells. In clinic, high circ-PRKCI in tumors was significantly correlated with lymph node metastasis and recurrence of PTC patients (Table 1).

Silencing circ-PRKCI suppressed cell growth, metastasis and glycolysis in PTC in vitro
SiRNAs targeting circ-PRKCI were used to silence circ-PRKCI expression in both TPC-1 and IHH-4 cells (Fig. 2A and Fig. S2). Comparing to si-NC transfection, both si-circ-PRKCI#1 and #2 transfection could retard cell proliferation of TPC-1 and IHH-4 cells, as indicated by MTT assay (Fig. 2B and 2C). Similarly, colony counts in transfected TPC-1 and IHH-4 cells were reduced in the presence of siRNAs targeting circ-PRKCI than its negative control (Fig. 2D). Downregulation of circ-PRKCI via siRNAs transfection compelled TPC-1 and IHH-4 cells to arrest in G0/G1 phase instead to enter in S phase, as evidenced by FACS data (Fig. 2E). Scratch wound assay and transwell assay showed less wound closure rate and invaded cells in si-circ-PRKCI (#1 and #2)-transfected TPC-1 and IHH-4 cells (Fig. 3A and 3B); moreover, expression of cell metastasis markers including MMP2, MMP3 and Snail was inhibited by administrating si-circ-PRKCI (#1 and #2) comparing to adding si-NC (Fig. 3C–3E). These results demonstrated that cell growth and metastasis of PTC cells in vitro were suppressed by silencing circ-PRKCI. Besides, glycolysis
was monitored, and the result was that glucose uptake and lactate production were lowered in circ-PRKCI-silenced TPC-1 and IHH-4 cells (Fig. 3F and 3G).

circ-PRKCI functioned as a sponge of miR-335 in PTC cells via target binding

According to circinteractome (https://circinteractome.nia.nih.gov/index.html) database, circ-PRKCI was 412 bp length spliced from PRKCI, and showed 18 miRNAs target sites, including miR-335 (Fig. 4A). Expression of miR-335 was lower in PTC tumor tissues than adjacent non-tumor tissues (Fig. 4B), and this expression was negatively correlated to circ-PRKCI (Fig. 4C). To validate this computational target relationship, miR-335 was abnormally overexpressed in TPC-1 and IHH-4 cells by transfecting miR-335 mimic (Fig. 4D), which significantly attenuated luciferase activity of reporter vector carrying wt-circ-PRKCI, and failed to reduce mut-circ-PRKCI vector’s luciferase activity (Fig. 4E and 4F); at the same time, circ-PRKCI could be largely enriched by Ago2 in TPC-1 and IHH-4 cells with ectopic expression of miR-335 (Fig. 4G and 4H). In TPC cells in vitro, miR-335 was downregulated than normal Nthy-ori 3-1 cells (Fig. 4I). Introducing circ-PRKCI overexpression vector induced circ-PRKCI upregulation and miR-335 downregulation (Fig. 4J and 4K); meanwhile, transfecting circ-PRKCI siRNAs prompted high expression of miR-335 (Fig. 4L), and this upregulation could also be diminished by adding miR-335 inhibitor (Fig. 4M and 4N). These results indicated that circ-PRKCI targeted and sponged miR-335 in PTC cells.

Downregulation of miR-335 counteracted the suppressive effect of circ-PRKCI deficiency on cell progression and glycolysis of PTC cells in vitro

Cell proliferation of TPC-1 and IHH-4 cells analyzed by MTT assay was inhibited by silencing circ-PRKCI, and this inhibition was abated by depleting miR-335 via its inhibitor transfection (Fig. 5A and 5B). Colony counts suppression in TPC-1 and IHH-4 cells and cell cycle arrest in G0/G1 phase mediated by circ-PRKCI deficiency were counteracted by additional miR-335 down-regulation, as depicted by colony formation assay and FACS method (Fig. 5C–5F). Likewise, blocking circ-PRKCI could descend wound closure rate, decrease transwell invaded cells, and depress expression of MMP2, MMP3 and Snail in TPC-1 and IHH-4 cells, which were overall abrogated with miR-335 inhibitor-induced downregulation of miR-335 (Fig. 5G–5L). Glucose uptake and lactate production in TPC-1 and IHH-4 cells were also suppressed when circ-PRKCI was knocked down, and this suppression was cancelled when circ-PRKCI and miR-335 were synchronously blocked (Fig. 5M and 5N). These data suggested that the suppressive role of circ-PRKCI deficiency in PTC cells was ascribed to the upregulation of miR-335.

E2F3 was positively regulated by circ-PRKCI in PTC cells via serving as a target gene of miR-335

A novel potential target gene of miR-335 was further
predicted according to starbase database (http://starbase.sysu.edu.cn/mRNA-miRNA target), and the computational binding sites in E2F3 were presented (Fig. 6A). E2F3 protein expression and mRNA expression were upregulated in PTC patients’ tumors than non-tumor tissues (Fig. 6B and 6C); in addition, Pearson correlation coefficient analysis analyzed an inverse correlation between E2F3 mRNA and miR-335 expression in PTC tumors (Fig. 6D). Ectopic expression of miR-335 not only attenuated luciferase activity of reporter vector carrying wt-E2F3 3’UTR (Fig. 6E and 6F), but also enriched E2F3 in Ago2-mediated RIP complex (Fig. 6G and 6H). Paralleled with that in PTC patients, E2F3 protein expression was higher in PTC cell lines (Fig. 6I) and was negatively regulated by miR-335 in TPC-1 and IHH-4 cells (Fig. 6J and 6K). Moreover, the inhibitory effect of miR-335 mimic on E2F3 expression could be alleviated in the presence of E2F3 overexpression vector (Fig. 6L and 6M). These outcomes proposed E2F3 as a direct target of miR-335 in PTC. Notably, si-circ-PRKCI#1 transfection-mediated circ-PRKCI silencing could also result in E2F3 downregulation, which was rescued by
adding miR-335 inhibitor (Fig. 6N); moreover, there was a positive correlation between E2F3 mRNA and circ-PRKCI expression in these PTC tumors (Fig. 6O). This finding hinted an interactive effect among circ-PRKCI, miR-335 and E2F3 in PTC.

**Overexpressing miR-335 inhibited cell growth, metastasis and glycolysis of PTC in vitro via depressing E2F3**

Ectopic expression of miR-335 deferred cell proliferation of TPC-1 and IHH-4 cells by transfecting its mimic (Fig. 7A and 7B). Colony formation ability and cell cycle progression were suppressed by miR-335 mimic comparing to mimic NC, as evidenced by the reduced colony counts, elevated G0/G1 phase cells, and declined S phase cells (Fig. 7C–7F). These data suggested a suppressive role of miR-335 overexpression in PTC cell growth in vitro, whereas this suppression could be partially reversed due to E2F3 overexpression via vector transfection (Fig. 7A–7F). Wound closure rate and transwell invasion of TPC-1 and IHH-4 cells were also depressed by upregulating miR-335 (Fig. 7G and 7H), accompanied with diminished levels of MMP2, MMP3 and Snail (Fig. 7I–7L). Similarly, restoring miR-335 inhibited PTC cell metastasis in vitro, which could be counteracted by restoring E2F3 as well (Fig. 7G–7L). Furthermore, excess administration of E2F3 overexpression vector could improve glycolysis of TPC-1 and IHH-4 cells with miR-335 overexpression, as described by the elevation of glucose uptake and lactate production (Fig. 7M and 7N). These outcomes together demonstrated a tumor-suppressive role of miR-335 in PTC cell progression and glycolysis in vitro via depressing E2F3.

**Depleting circ-PRKCI retarded cell growth of PTC in vivo by regulating E2F3 and miR-335**

TPC-1 cells were inoculated in nude mice, and xenograft tumors were generated. shRNAs injection was...
performed from the 4th day after cell inoculation, and thus tumor growth in successive days was retarded in sh-circ-PRKCI group (n = 5) comparing to sh-NC group (n = 5), as exhibited by the lowered tumor volume and weight (Fig. 8A–8C). Expression of circ-PRKCI in xeno-graft tumor tissues was lower in sh-circ-PRKCI group (n = 5), accompanied with promoted miR-335 and depressed E2F3 (Fig. 8D and 8E).

Discussion

Growing evidences had demonstrated that circRNAs possessed potent potency of biomarkers and therapeutic targets for PTC [22], such as circRAPGEF5, hsa_circ_0058124 and hsa_circ_0006156 [23, 24]. PRKCI could generate multiple circRNAs, among which there was a well-studied circRNA, namely hsa_circ_0067934. Exhaustion of hsa_circ_0067934 could repress cell progression of several cancers, including esophageal cancer, glioma, and lung cancer [25-27], as well as thyroid cancer [28]. Nevertheless, hsa_circ_0122683 referred as circ-PRKCI in this study was left to be further annotated. Moreover, Liu et al. [12] analyzed GSE93522 dataset and found that circ-PRKCI was upregulated in PTC tissues than matching contralateral normal tissues. Therefore, we explored the biological role and ceRNA...
mechanism of this circRNA in PTC cell growth, metastasis and glycolysis.

Here, circ-PRKCI was upregulated in human TPC tissues and cells, and this upregulation was associated with lymph node metastasis and recurrence in these TPC patients. Moreover, circ-PRKCI predominantly located in cytoplasm with resistance to RNase R digestion and Act D degradation. In function, blocking circ-PRKCI could arrest cell cycle and suppress cell proliferation, colony formation, migration, invasion, and glycolysis of TPC cells in vitro, as well as inhibit cell growth in vivo. Expression of metastasis-related proteins including MMP2, MMP3 and Snail was downregulated in response to circ-PRKCI knockdown, either. However, the clinical data showed that circ-PRKCI expression was not significantly correlated with some clinicopathological factors of PTC patients, such as tumor size, TNM staging and extrathyroid extension. This discrepancy between these clinical findings and experimental findings regarding circ-PRKCI expression role in PTC cell growth, migration.

Fig. 5 Downregulation of miR-335 counteracted the effect of circ-PRKCI deficiency in TPC cells. (A–N) TPC-1 and IHH-4 cells were severally transfected with si-NC, si-circ-PRKCI#1, si-circ-PRKCI#1 and miR-335 inhibitor, si-circ-PRKCI#1 and NC inhibitor for 48 h. (A, B) MTT assay monitored OD values of transfected cells for another 3 days. (C, D) Colony formation assay showed colonies formed in transfected cells for another 14 days, and colony counts were determined. (E, F) FACS determined the percentage of cell cycle distribution. (G) Scratch wound assay measured wound closure rate of transfected cells for another 24 h. (H) Transwell assay determined the number of invaded cells in transfected cells for another 48 h. (I–L) Western blotting measured expression of MMP2, MMP3 and Snail proteins with normalization to β-actin. (M, N) Glucose and lactate assay kits evaluated the percentage of glucose uptake and lactate production. * p < 0.05.
and invasion might due to the small patient sample and idealized controlled conditions in laboratory.

Besides, downregulation of hsa_circ_0067934 also exhibited tumor-suppressive role in TPC by curbing cell proliferation, migration, invasion and tumor growth [28]. These results suggested that circ-PRKCI and hsa_circ_0067934 (both derived from PRKCI) were onco-genic in TPC carcinogenesis, development and treatment. This study might provide a new avenue to the diagnosis and treatment strategy for TPC.

Similarly, circ-PRKCI was identified to directly sponge miR-335 in the regulation of TPC cell progression and glycolysis. According to miRNA microarray, miR-335 was the most downregulated miRNA in PTC tumors throughout stage I to III [29]; besides, miR-335 together with miR-130a, miR-126, miR-374b and miR-486-5p was lower in stage II-III tumors and higher in stage I tumors than paired adjacent non-tumor tissues.

**Fig. 6** E2F3 was positively regulated by circ-PRKCI via serving as target gene of miR-335. (A) The miR-335-binding sites in wt-E2F3 3’UTR were mutated to construct mut-E2F3 3’UTR. (B, C) Western blotting and qPCR detected E2F3 protein expression and mRNA expression in tissues from TPC patients. N represented adjacent non-tumor tissue, and T represented TPC tumor tissue. (D) Pearson correlation coefficient analysis analyzed the correlation between E2F3 mRNA and miR-335 expression in TPC tumors. (E, F) Dual-luciferase reporter assay measured luciferase activity of reporter vectors (wt-E2F3 3’UTR and mut-E2F3 3’UTR) in TPC-1 and IHH-4 cells transfected with miR-335 mimic or NC mimic. (G, H) RIP assay determined the enrichment of E2F3 mRNA by Ago2 or IgG in TPC-1 and IHH-4 cells. (I–N) Western blotting and qPCR detected E2F3 protein expression in (I) Nthy-ori 3-1, TPC-1, IHH-4, and SNU-790 cells, (J–N) TPC-1 and IHH-4 cells transfected with NC mimic, miR-335 mimic, NC inhibitor, miR-335 inhibitor, pCDNA3.1-E2F3 vector (E2F3), pCDNA3.1 vector, miR-335 mimic and pDNA3.1. miR-335 mimic and E2F3, si-circ-PRKCI#1 and miR-335 inhibitor, si-circ-PRKCI#1 and NC inhibitor. (O) Pearson correlation coefficient analysis analyzed the correlation between E2F3 mRNA and circ-PRKCI expression in TPC tumors. * p < 0.05.
In this study, our data indicated a downregulation of miR-335 in clinic TPC samples, and this expression seemed to be not correlated with TNM staging. Furthermore, it had been previously demonstrated that miR-335 was downregulated in PTC tissues and cells, and that its overexpression mediated anti-growth, anti-migration and anti-invasion role in PTC [17, 18, 30]. Instead of performing transwell assay, we executed scratch wound assay to measure cell migration ability. Zhang et al. [29] proposed dozens of computational target genes for miR-335, and many of which were involved in cell proliferation, differentiation, apoptosis, cell cycle, and signaling transduction pathway. In addition, intercellular adhesion molecule 1 (ICAM-1; a molecule that was conductive to invasion and metastasis) and SHH (a key factor that was involved in Hedgehog pathway) had been validated to be downstream target genes for miR-335 [18, 30]. Here, E2F3, as oncogene with strong prolifera-

Fig. 7 Upregulation of E2F3 counteracted the effect of miR-335 overexpression in TPC cells in vitro. (A–N) TPC-1 and IHH-4 cells were severally transfected with NC mimic, miR-335 mimic, miR-335 mimic and pcDNA3.1, miR-335 mimic and E2F3 for 48 h. (A, B) MTT assay monitored OD values of transfected cells for another 3 days. (C, D) Colony formation assay showed colonies formed in transfected cells for another 14 days, and colony counts were determined. (E, F) FACS determined the percentage of cell cycle distribution. (G, H) Scratch wound assay and transwell assay measured wound closure rate and invaded cell number in transfected cells for another 24 h and 48 h, respectively. (I–L) Western blotting measured expression of MMP2, MMP3 and Snail proteins with normalization to β-actin. (M, N) Glucose and lactate assay kits evaluated the percentage of glucose uptake and lactate production. *p < 0.05.
tive potential [16], had been newly discovered to be targeted by miR-335.

pRb/E2Fs was mutated in almost all human tumors [31], and E2F3 was required in the development and tumorigenesis of pRb-deficient thyroid tumors [32]. There were two distinct E2F3 proteins: E2F3a and E2F3b [16]. Here, we focused on E2F3a which could drive cell cycle progression [33]. E2F3a could be targeted and regulated by several miRNAs in regulating human malignant tumors, such as miR-34a [34, 35] and miR-128 [36]. In this study, bioinformatics algorithms of starbase software presented potential miR-335-binding sites in both E2F3a (related to ENST00000346618) and E2F3b (related to ENST00000535432); however, we just further confirmed the target relationship between miR-335 and E2F3a. Functionally, restoring E2F3 could rescue miR-335-mediated suppression on proliferation, colony formation, cell cycle progression, migration, and invasion, as well as glycolysis. Very recently, Zang et al. [37] observed an association between E2F3 and glycolysis in breast cancer.

Collectively, we demonstrated that silencing circ-PRKCI suppressed PTC by inhibiting cell growth, metastasis and glycolysis via circ-PRKCI/miR-335/E2F3 ceRNA pathway. This study firstly annotated the oncogenic role of circ-PRKCI in PTC cells and its potential application in PTC clinical diagnosis for metastasis and recurrence. Moreover, the circ-PRKCI/miR-335/E2F3 ceRNA pathway was confirmed to be underlying PTC carcinogenesis. Furthermore, this work might disclose the role of miR-335 and E2F3 in the field of glycolysis in PTC cells.

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Disclosure of Interest
The authors declare that they have no financial conflicts of interest.

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Ethics Approval and Consent Participate
Written informed consent was obtained from patients with approval by the Institutional Review Board in Guizhou Provincial People’s Hospital.
**Fig. S1**  The effect of DMSO on RNA expression in PTC cells. (A, B) qPCR detected circ-PRKCI and PRKCI mRNA levels in TPC-1 and IHH-4 cells treated with 0.1% DMSO. *p < 0.05.

**Fig. S2**  The E2F3 protein expression in PTC cells with siRNAs transfection. Western blotting detected E2F3 level in TPC-1 and IHH-4 cells transfected with si-NC, si-circ-PRKCI#1 or si-circ-PRKCI#2. *p < 0.05.

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